Studies of infectious pancreatic necrosis virus (IPNV) and immune evasion strategies

Astrid Skjesol

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UNIVERSITY OF TROMSØ
Faculty of Biosciences, Fisheries and Economics
Norwegian College of Fishery Science

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Astrid Skjesol, July 2009

Contents

Abbreviations

ADAR	Adenosine deaminase acting on RNA
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma 2
BF-2	Bluegill sunfish fibroblastic cells
BSNV	Blotched snakehead virus
CARD	Cardif adaptor inducing IFN-β
CBP	CREB-binding protein
$CD4^+$ Th1	Cluster of differentiation 4 positive T-helper lymphocyte
CHSE-214	Chinook salmon embryo cells
CpG	Cytosine-phosphate-guanine
CRFB	Class II helical cytokine receptors
DBD	DNA-binding domain
DNA	Deoxyribonucleic acid
dsRNA	double-stranded RNA
DXV	Drosophila X virus
eIF-2	Eukaryotic translation initiation factor 2
ELISA	Enzyme linked immunosorbent assay
EPC	Carp epithelial cells
ERK	Extracellular signal-regulated kinase
GAS	Gamma interferon activation site
GIG2	Gamma inducible gene 2
GTP	Guanosine triphosphate
h p.i.	Hours post infection
HAT	Histone acetyl transferase
HBV	Hepatitis B virus
HCV	Hepatitis C virus
IBDV	Infectious bursal disease virus
IFN	Interferon
IFNAR	Interferon α/β receptor
IFNGR	Interferon γ receptor
IHN	Infectious hematopoietic necrosis
IHNV	Infectious hematopoietic necrosis virus
IKK	inhibitor of NF-κB kinase

IL	Interleukin
IPN	Infectious pancreatic necrosis
IPNV	Infectious pancreatic necrosis virus
IPS-1	IFN-β promoter stimulator protein 1
IRAK	IL-1 receptor-associated kinase
IRF	Interferon regulatory factor
ISA	Infectious salmon anemia
ISAV	Infectious salmon anemia virus
ISG	Interferon stimulated gene
ISGF3	Interferon stimulated gene factor 3
ISRE	Interferon stimulated response element
IκB	Inhibitor of NF-κB
JAK	Janus activated kinase
kb	Kilobase
kDa	Kilodalton
MALDI/TOF	Matrix assisted laser desorption ionization/time of flight
MAPK	Mitogene-activated protein kinase
MAVS	Mitochondrial antiviral signaling
Mda5	Melanoma differentiation associated gene
MHC	Major histocompatability complex
mRNA	Messenger RNA
MS	Mass spectometry
Mx	Myxovirus resistance
MyD88	Myeloid differentiation primary response gene 88
NEMO	NF-κB essential modulator
NES	Nuclear export signal
NF-κB	Nuclear transcription factor-kappaB
NK	Natural killer cells
NLS	Nuclear localization signal
nm	Nanometer
NS	Non-structural
OAS	2', 5'-Oligoadenylat synthetase
ORF	Open reading frame
PAMP	pathogen-associated molecular pattern
PCR	Polymerase chain reaction

pDC	Plasmacytoid dendrittic cell
PIAS	Protein inhibitor of activated STAT1
PKR	Protein kinase R
Poly I:C	Polyinosinic polycytidylic acid
PRR	Pattern recognition receptor
qPCR	quantitative PCR
RdRp	RNA-dependent RNA polymerase
RIG-I	Retinoic acid inducible gene I
RNA	Ribonucleic acid
RNAi	RNA interference
RT-PCR	Reverse transcription polymerase chain reaction
SH2	Src homology 2
SOCS	Supressor of cytokine signaling
Sp1	Ubiquitous cellular transcription factor
ssRNA	Single stranded RNA
ssSTAT	Salmo salar Signal transducers and activators of transcription
STAT	Signal transducers and activators of transcription
SV5	Simian virus 5
TAD	Transcriptional activation domain
TANK	TRAF family member-associated NF-κB activator
TBK1	TANK-binding kinase 1
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TO	Atlantic salmon head kidney cell-line
TRAF	Tumor necrosis factor receptor-associated factor
TRIF	TIR-domain-containing adapter-inducing interferon-β
TYK	Tyrosine kinase
UBL	Ubiquitin-like
VHSV	Viral hemmorhagic septicaemia virus
VISA	Virus-induced signaling adaptor
VOPBA	Virus overlay protein binding assay
VP	Virus protein
VSV	Vesicular stomatitis virus
Y2H	Yeast two-hybrid system

Sammendrag (summary in Norwegian)

Infeksiøs pankreas nekrose (IPN) er en smittsom sykdom som rammer salmonider i tidlige livsstadier. Økonomiske tap som følge av sykdommen er et problem for kommersielle oppdrettere av laks, ørret og røye over store deler av verden. Viruset som forårsaker denne sykdommen, infeksiøst pankreas nekrose virus (IPNV), er et dobbeltrådig RNA (dsRNA) virus i familien Birnaviridae. Viruset har en ikosahedrisk kapsid-struktur, omtrent 60 nm i diameter, og er uten en ytre kappe. Virusgenomet består av to dsRNA segmenter, kalt segment A og B, som koder for fem proteiner, VP1-VP5. VP1 er den virale RNA-polymerasen som kodes av Segment B. Dette proteinet finnes i to former – fritt eller bundet til dsRNA. På segment A er det en stor åpen leseramme som koder for et polyprotein som kløyves av virusets egen protease, VP4, for å danne strukturproteinene VP2 og VP3. IPNV kapsidet, som er virusets ytre struktur, er bygd opp av VP2. I tillegg har segment A en liten, alternativ leseramme som koder for VP5, et ikke-strukturelt protein med hittil udefinerte egenskaper.

Dagens vaksiner gir en begrenset beskyttelse mot IPNV. For å optimalisere effekten av vaksinering, er det viktig å få en bedre forståelse for hvordan spesifikke faktorer i virus og vert interagerer med hverandre under en infeksjon. I denne avhandlingen beskrives fysiske interaksjoner mellom ulike virusproteiner og betydningen av disse diskuteres. Ved å benytte nye molekylærbiologiske metoder har vi vist at VP3 har en sentral rolle i viruspartikkelen fordi det binder seg både til andre VP3 proteiner, til VP1 polymerasen og til virusgenomet. De ulike domenene for binding er kartlagt.

Det er også viktig å kartlegge hvordan verten forsvarer seg mot virusinfeksjoner. Dette er lite studert hos fisk. Interferoner er alarmproteiner som produseres når virus infiserer mennesker og dyr. Dette forsvarssystemet slås på av virusets arvestoff (RNA) og kan stoppe videre invasjon av viruset. Det finnes flere typer IFN. Type I IFN (IFNα/β) er det som primært er assosiert med direkte antiviral aktivitet, mens type II (IFNγ) i tillegg har mer spesialiserte funksjoner knyttet til ervervet immunitet. Gjennom evolusjonen har mange virus utviklet strategier for å unngå vertens forsvar mot virus. Dette gjelder også for IPNV. Tilsetning av IFN gjør uinfiserte celler svært motstandsdyktige mot IPNV. Da evner ikke viruset å gjennomføre egen proteinsyntese og dannelsen av nye viruspartikler blokkeres. Hos celler som er infisert med IPNV før de behandles med IFN, klarer IPNV ”å slå tilbake” slik at uttrykket av antivirale gener i vertscella dempes. Det ser altså ut til at viruset har strategier for

å hemme signalveien nedstrøms for IFN, den såkalte JAK/STAT signalveien. Vi foreslår at VP4 og VP5 er kandidater til denne "motstandsfunksjonen".

STAT1 er et viktig molekyl som aktiveres av IFN og bidrar til oppregulering av antivirale gener. I dette arbeidet har vi klonet STAT1 fra atlantisk laks og vist at i likhet med pattedyr vil laksens STAT1 bli aktivert i IFN-stimulerte celler. Vi viser at også laksens STAT1 har et konservert tyrosin som fosforyleres på signal fra både type I og type II IFN og transporteres fra cytoplasma inn til cellekjernen på signal fra IFNγ. I tillegg har vi sett at STAT1 danner dimerer med andre STAT1 molekyler. Vi har ikke funnet noen direkte interaksjon mellom IPN virus-proteinene og STAT1 som kan forklare virusets evne til å hemme IFN-responsen. Vi vil arbeide videre med å kartlegge hvilke molekyler som er involvert i denne effekten

List of papers

Paper 1

Torunn Pedersen, Astrid Skjesol and Jorunn B. Jørgensen (2007)

VP3, a structural protein of the infectious pancreatic necrosis virus, interacts with the RNA-dependent RNA polymerase VP1 and with double-stranded RNA. Journal of Virology 81(12): 6652-6663.

Paper 2

Astrid Skjesol, Toril Aamo, Marit Nøst Hegseth, Børre Robertsen and Jorunn B. Jørgensen

The interplay between infectious pancreatic necrosis virus (IPNV) and the IFN system: IFN signaling is inhibited by IPNV infection. Virus Research 143(1): 53-60.

Paper 3

Astrid Skjesol, Tom Hansen, Cheng-Yin Shi, Hanna Leena Thim and Jorunn B. Jørgensen

Structural and functional studies of the STAT1 gene from Atlantic salmon. Manuscript.

Introduction

Viruses are intracellular, parasitic particles that depend on the host cells they infect in order to reproduce. Viruses consist of either a single-stranded or a double-stranded DNA or RNA genome in addition to a protective protein coat. Viruses exist in the air, soil and water and infect all cellular life. As protection against the dozens of viruses that attack, vertebrates have two major ways to defend themselves: a general antiviral protection provided mainly by small molecules called interferons and specific mechanisms like antibody production and killing of virus-infected cells by cytotoxic T cells. Most antibodies give lifelong immunity, but it takes days or weeks to produce them, and each type of antibody is effective against only one type of virus. Interferon production is faster, and occurs within a few hours of exposure, but still it is not fast enough to keep many invading viruses from multiplying in millions of cells and causing severe illness. Interferons are effective against the whole catalogue of viruses, but its protection may last only a short period. Thus the immune system is a collection of biological processes within an organism that protects against disease by identifying and fighting pathogens in clever ways.

Infectious pancreatic necrosis (IPN)

History of IPN

Infectious pancreatic necrosis (IPN), previously called acute catarrhal enteritis, is a severe, contagious disease mainly occurring among salmonids. The disease was first described in the United States in 1941 (M'Gonigle, 1941), but was not detected in Norway until 1971. Early descriptions of the occurrence of the disease were usually connected to fry in the early fresh-water phase and with high mortality numbers (Wood et al., 1955). Later, from the mid-1980s, outbreaks have often been reported to occur during transfer from fresh- to sea-water (smoltification) and increasingly, also among adult fish.

Pathology of IPN

Pathological changes include abnormal, whirling swimming motion, darkening pigmentation, pronounced distended abdomen, necrotic tissue in pancreas, milky mucus in stomach and anterior intestine, long thin whitish faecal casts and pale gills, spleen, kidney, liver and heart (OIE, 2000).

Epidemiology of IPN

The causative agent is the IPN virus (IPNV) which has a history as one of the major loss factors in Norwegian and international (salmonid) aquaculture. IPNV has been isolated from economically important salmonid species such as trout, salmon, whitefish, and grayling, and additionally from many other fish species including, herring, sole, halibut, cod, saithe, eel, bass, pike, perch, carp, cichlids and lamprey (Noga, 2000). Some of these are also potential aquaculture species. Aquatic invertebrates such as oysters, crabs and even trematodes have also been found to be infected with IPNV. To these nonsalmonid species IPNV is only occasionally being pathogenic, meaning that in a clinical aspect these species function as carriers or reservoirs of virus isolates (Noga, 2000). IPNV was for the first time, as the first fish pathogen, isolated and propagated in cell culture in 1958 (Wolf et al., 1958) and has later been extensively studied. As Atlantic salmon aquaculture has dramatically expanded, the disease has emerged worldwide and is now recognized to be the economically most important disease in salmon production in the European Union and in Norway (Roberts and Pearson, 2005). A comprehensive survey of more than 30 000 fish comprising 37 species in the Scottish marine environment has been undertaken by Wallace et al. (2008) in order to investigate the prevalence of IPNV in wild fish near clinically infected Atlantic salmon farms. This study shows that there is a localized small increase in the prevalence of IPNV in wild marine fish caught near the farms. Fortyfive isolations of IPNV were made from nine different species. The results suggest that farms do act as a source of infection to wild fish; however, there is also evidence that IPNV is endemic in wild marine fish. Wild fish reservoirs are, however, probably not a major factor for infection of marine salmon farms (Murray, 2006). In addition to horizontal transmission, IPNV has been shown to be vertically transmitted through the germline from parent to progeny in rainbow trout (Dorson and Torchy, 1985). This process is also strongly suspected to occur in Atlantic salmon, though it has not yet been proven experimentally (Smail and Munro, 2008).

Prevention and control of IPN

In general, viral disease can largely be prevented by rearing fish in a good environment, with good nutrition, a minimum of stress, and isolated from sources of infectious agents. Avoidance of pathogens requires good sanitation at the fish farms and is best achieved with a pathogen-free water supply, the use of certified pathogen-free stocks, and strict biosecurity. Development of disease also depends on a complex set of factors such as interactions among the host, the pathogen, and the environment. Host factors include the species, size or age,

strain or stock, immune status, and general physiological condition. For the pathogen, factors include the concentration of infectious particles present in water, their physiological properties and the virulence of the strain. The difference between apparent health and disease typically depends on the balance between the pathogen and the host, and that balance is greatly influenced by environmental factors such as temperature and water chemistry. There is no reliable treatment for IPN, therefore disinfection and quarantine are the best methods of controlling IPN outbreaks.

Screening

Broodstock testing is very important for fish farmers to limit spread of disease. Currently, screening for diseases is based upon isolation of the virus in tissue culture followed by immunological identification. Additionally, reverse transcription-polymerase chain reaction (RT-PCR) protocols for IPNV have been developed (Blake et al., 1995; Lopez-Lastra et al., 1994; Taksdal et al., 2001). A very high proportion of macrophages in kidney tissue and blood leucocytes from IPNV-carriers are infected with IPNV (Munro et al., 2006). Thus, destructive kidney sampling followed by PCR and cell-based viral titration is the most sensitive method for detecting Atlantic salmon broodfish carrying IPNV (Munro and Ellis, 2008). New, diagnostic methods for rapid and reliable detection and identification of IPNV, based on RT-PCR-ELISA (Milne et al., 2006) and reverse transcription loop-mediated isothermal amplification (RT-LAMP) technology (Soliman et al., 2009) have been proposed. Offering non-lethal detection methods will be useful and more cost-efficient to the industry. Sampling based on culturing of adherent blood moncytes or gonadal fluids are less sensitive, but beneficial in that they are non-lethal detection methods. A highly sensitive and specific real-time RT-PCR assay has recently been developed for the detection and quantitation of IPNV in rainbow trout. The viral protease, VP4, can reliably be detected down to 10 RNA copies in spleen and head kidney as early as 24 h post-challenge, and non-lethal sampling from pectoral fin gives the same results (Bowers et al., 2008). The source of the virus in the case of vertical transmission of IPN is likely to be the sperm of carrier males or female carrier eggs, meaning that this method is also beneficial to facilitate investigations into the possibility of vertical transmission of IPNV in Atlantic salmon (Smail and Munro, 2008).

Table 1. Outbreaks of IPN, ISA, PD and VHS reported in Norwegian fish farms in the years 2002-2008 (Report on the health status in Norwegian fish farms 2008, by the National Veterinary Institute).

	2002	2003	2004	2005	2006	2007	2008
IPN	174	178	172	208	207	165	158
ISA	12	8	16	11	4	7	7
PD	14	22	43	45	58	98	108
VHS	0	0	0	0	0	3	2

Vaccines

IPN vaccines have been developed and a vaccination program has been ongoing since 1995, although the efficacy of the current vaccines is debatable. In Norway, 85% of smolts are given the vaccine; still, more outbreaks are reported due to IPNV than for any other fish pathogen every year. In 2008 158 fish farms along the coast had outbreaks of IPN (www.ssb.no) (Table 1). The vaccines against IPNV currently commercially available in Norway are multivalent bacterin oil-adjuvanted vaccines based on a recombinantly expressed subunit of the IPN virus (Frost and Ness, 1997) or formalin inactivated IPNV (Pharmaq, Novartis). No live attenuated vaccines or DNA vaccines are currently licensed in Europe, but one DNA vaccine against the rhabdovirus infectious hematopoietic necrosis virus (IHNV) has been licensed in Canada (July 2005, Apex-IHN, Novartis) after controlled field trials (Salonius et al., 2007). A DNA vaccine which is protective against another rhabdovirus, VHSV (Lorenzen and LaPatra, 2005), is currently being evaluated for use in Denmark. Survivors of IPNV infection usually become persistently infected and thereby serve as pathogen reservoirs (McAllister et al., 1987). IPNV is also strikingly environmentally persistent and can survive for days and months in water and even in air (Toranzo and Hetrick, 1982). It has also been shown that prophylaxis against IHNV in rainbow trout is actually induced by pre-exposure to a non-lethal infection with IPNV, probably due to induction of interferons and thereby establishment of an antiviral state (Kim et al., 2009; Saint-Jean and Pérez-Prieto, 2007).

Genetic selection by breeding

Vaccine trals with IPNV have shown that the genetic constitution of the fish has a strong influence on the protective outcome of the vaccine (Ramstad and Midtlyng, 2008). Although this variability has provided a problem in achieving high and consistent control-fish

mortalities in challenges for vaccine testing, the hereditable, genetic resistance to IPNV can be successfully used in Atlantic salmon family breeding schemes. Genetic information on family differences in resistance has been collected from a large challenge study with IPNV in Atlantic salmon post-smolts (Guy et al., 2006). Using a genome-wide scan, the most significant quantitative trait locus (QTL) found was mapped to LG 21. QTLs are stretches of DNA that are closely linked to the genes that underlie the inheritance of a phenotypic characteristic in question. This knowledge may be developed for use in selection for breeding strains of Atlantic salmon with greater resistance to IPN (Houston et al., 2008). One should be avare though, that intensive selection of one attribute might cause loss of other desired traits.

IPNV

Classification

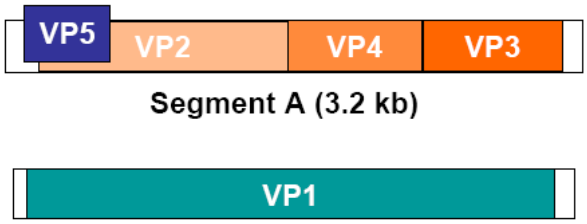
IPNV is classified as an Aquabirnavirus within the family *Birnaviridae.* Other members are the infectious bursal disease virus (IBDV), an Avibirnavirus that infects young chickens, and drosophila X virus (DXV) of the genus Entomobirnavirus, which is infectious to the fruitfly *Drosophila melanogaster* (Dobos 1995). In addition to IPNV, the genus Aquabirnavirus includes Yellowtail ascites virus, Tellina virus and a group of unclassified Aquabirnaviruses, including marine birnavirus and Japanese aquabirnavirus (NCBI taxonomy browser). Most knowledge about the birnaviruses is based on studies of IBDV and IPNV (Muller et al., 2003; Rodriguez Saint-Jean et al., 2003).

IPNV genome and proteins

The common feature for members of the *Birnaviridae* family is their bi-segmented dsRNA genome (designated segment A and B) that is enclosed in a non-enveloped single-shelled icosahedral particle of 60 to 70 nm in diameter (Figure 1). VP1, the viral RNA-dependent RNA polymerase (RdRp) encoded by segment B, is the largest of the viral proteins with a mass of 94 kDa which comprices only 4% of the virion. VP1 is found in both free and genome-linked forms in the virion. The genome-linked form, VPg, is linked by a phosphodiester bond to the 5'-end of both genome segments (Dobos, 1995). The larger of the two segments, segment A (3.1 kb), encodes a polyprotein which is cotranslationally cleaved by the viral encoded serine-lysine protease (VP4) releasing proteins pVP2 and VP3 (Duncan and Dobos, 1986; Duncan et al., 1987). pVP2 is further processed most efficiently by host cell proteases to form the mature outer capsid protein VP2 (Magyar and Dobos, 1994b), which

makes up about 60% of the virion (Dobos, 1995) and contains the antigenic region responsible for induction of neutralizing antibodies in the host (Heppell et al., 1995). The viral subunit vaccine currently available in Norway is based on the epitope-containing amino acids 86-210 of VP2 recombinantly expressed in *E. coli* (Christie, 1997; Frost et al., 1995; Frost and Ness, 1997). VP3 is an internal structural protein which is the protein present in highest numbers in the virion, although with a smaller size than VP2 (27 kDa vs 49 kDa) it constitutes only 34% of the virion (Dobos, 1995). In IBDV an interaction between VP3 and VP1 has been reported to be important for assembly of the virus particle. IBDV VP3 also interacts with itself and binds to viral RNA predicting its function to be a multitasking organizer (Maraver et al., 2003; Tacken et al., 2002). As mentioned, IPNV VP4 is the viral protease critical for autocleavage of the polyprotein encoded by segment A. Two amino acid residues, serine 633 and lysine 674 are critical for its cleavage activity (Petit et al., 2000). These residues are conserved across the Lon family of proteases (Birghan et al., 2000). VP4 was recently crystallized, and four substrate binding pockets identified, providing insights into the catalytic mechanism of this protease (Lee et al., 2007). Segment A contains a second, smaller open reading frame in the 5' end, which encodes the non-structural protein, VP5 (Magyar and Dobos, 1994a), with so far undefined properties. Hong et al.(2002) have suggested an anti-apoptotic effect of VP5, based on its homology to the anti-apoptotic protein Bcl-2, and enhanced cell viability by over-expression of VP5 *in vitro*. This property is contradicted by Santi et al. (2005a), who fail to show antiapoptotic activity of VP5. These conflicting results are suggested to be due to substitutions of amino acids in the domain being functionally important for the anti-apoptotic effect of the protein. Later, IPNV has been shown to activate the apoptosis-regulating transcription factor NF-κB, possibly through the tyrosine kinase pathway (Hong et al., 2008). There is extensive homology between the noncoding sequences of segment A and B which is suggested to be important for polymerase recognition, translation initiation and possibly genome packaging (Dobos, 1995).

A



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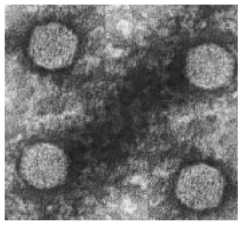

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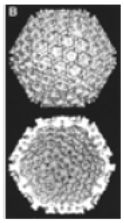


D

Proteins	Mw (kDa)	Function
VP1	94	RNA-dependent RNA polymerase
VP2	51	Structural protein: outer capsid
VP3	27	Inner structural protein
VP4	24	Cis-acting protease
VP5	15	Non-structural protein, unknown function. Antiapoptotic?

Figure 1: A. The two genomic segments of IPNV encode 5 proteins. **B.** Electronmicrography of IPNV particles (from (Villanueva et al., 2004)). **C.** Model of IBDV. **D.** Table showing the molecular weight (Mw) in kilo Daltons (kDa) and the best characterized function of each viral protein.

Viral replication

The full IPNV replication cycle takes about 24 h at 15°C, or 16-20 h at 22°C in Chinook salmon embryo (CHSE-214) cells (Dobos, 1995; Lannan et al., 1984). IPNV, like other nonenveloped animal viruses, seems to enter the host cells by receptor mediated endocytosis after specific attachment to CHSE-214 cells (de las Heras et al., 2008; Granzow et al., 1997; Kuznar et al., 1995) (Figure 2). Specific attachment of IPNV to other fish cell types (BF-2 and EPC) is also observed (de las Heras et al., 2008). VP2 is suggested to be the cell attachment protein (Dobos, 1995), but no cellular recognition molecule has been identified. After cell entry, the IPNV may proceed directly with transcription as uncoating is not necessary and the RdRp is active without further proteolytic treatment. The replication cycle takes place in the cytoplasm (Cohen, 1975). Transcription is primed by VP1 binding to RNA followed by a semi-conservative strand-displacement on the positive RNA-strand (Dobos, 1995). Recent studies have shown that the negative RNA strand is the template for the genomic replication (Cortés-San Martin et al., In press). Viral mRNA can be detected as early as 4-6 h post infection (p.i.), and shortly after, virus specific polypeptides occur. The synthesis of viral RNA reaches its peak level 8-10 h p.i. (Cortés-San Martin et al., In press;

Dobos, 1995). During a single replicative cycle, two different kinds of particles are formed; one larger, uninfectious particle termed provirion, where the capsid is composed of both mature and immature viral polypeptides, the other is the mature, infectious virion (Villanueva et al., 2004). It is uncertain how the viral progeny is released from the cells, as no exocytosis or other virus releasing mechanisms have been observed (Granzow et al., 1997).

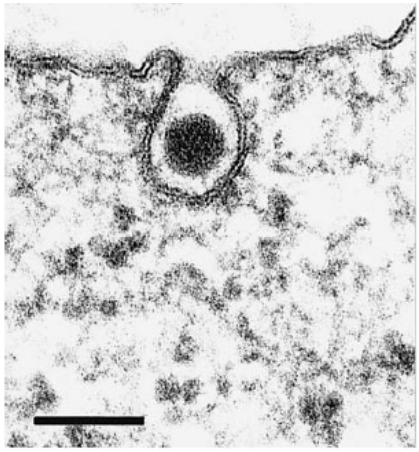


Figure 2: IPNV enters into a rainbow trout gonad (RTG-2) cell by endocytosis. Electronmicrography by (Granzow et al., 1997) Bar = 100 nm.

Virulence factors

The mortality of IPN outbreaks in Atlantic salmon farming varies considerably. Mortality rates ranging from 6 – 90% are reported from field outbreaks (Santi et al., 2004). The viral genome is under strong selection pressure exerted by the host immune system. The molecular basis for the variation in virulence has been studied by genomic sequencing of IPNV isolates collected from outbreaks of the disease in Norwegian fish farms. Differences are revealed in the genomic sequences of VP2 (Blake et al., 2001; Santi et al., 2004), VP3 and VP5 ((Santi et al., 2004) own unpublished data). Certain amino acids in the sequence of VP2 have proven to be important factors in the determination of the virulence of a strain. Threonin (T), alanin (A) and T in positions 217, 221 and 247, respectively, gives a highly virulent virus. In low virulence strains these positions are exchanged with proline (P)AT (Santi et al., 2004). The amino acid residue 221 of VP2 is in addition identified as a possible persistence determinant. Strains encoding threonine at this position establish a carrier state in almost 100% of the fish, while fish challenged with strains encoding alanine at this position either cleared the infection, or became carriers of a virus with a mutation in VP2 residue 221 (Song et al., 2005). Also VP5 has been suggested as a virulence factor. However, a study applying recombinant, truncated variants of VP5 induced more than 80% cumulative mortality excluding VP5 as an important virulence factor in IPNV infection (Santi et al., 2005b). VP5 has also been shown to be dispensable for the establishment of a carrier state (Santi et al., 2005b). Interestingly, IPNV cause viral interference to replication of IHNV, which show significant reduction in titers when co-infected with IPNV. The mechanisms through which IPNV interferes with IHNV

replication are poorly understood and might be due to competition for viral receptors, unspecific activation of immune responses or accumulation of dsRNA (de las Heras et al., 2008). Other factors may apply to the virus's ability to replicate in host cells. Further genomic analyses linked to the virus's virulence in addition to comparative studies of virulent and non-virulent isolates and their effect on host gene-expression (i.e. microarrays and RT q-PCR) can elucidate possible genetic traits that lead to virulence. The roles of the individual virus proteins connected to protection, persistence and virulence are still not properly resolved.

Interferons

Interferon (IFN) was discovered more than 50 years ago as an agent that inhibited the replication of influenza virus (Isaacs and Lindenmann, 1957). The IFN family of cytokines is now recognized as key components of the innate immune system. Accordingly, IFNs are currently being used therapeutically, with antiviral effects in the treatment of some viral diseases and they also show effectiveness in suppressing certain cancers (Borden et al., 2007). There are three types of IFN described in mammals (type I-III). Animal viral infection directly induces type I IFN (IFN α/β/κ/δ/ε/τ/ω/ζ) or the recently described type III IFN (IFNλ1/2/3) synthesis (Onoguchi et al., 2007). The synthesized and secreted cytokines in turn induce transcription of antiviral genes through a signaling cascade as part of the "first-wave" immune response of the innate immune system, acting within hours, whereas adaptive immune responses like antibody production takes days (Samuel, 2001). Type II IFN has a single member; IFNγ which is secreted mainly by activated T cells and natural killer (NK) cells, rather than in direct response to viral infection. Although IFNs have crucial roles in regulating innate immune responses, they are also factors in adaptive immunity.

Ways to identify "the bad guys"; IFN induction/production

There are several ways in which a cell can recognize an infectious agent, and multiple signaling pathways that can lead to induction of IFNs (Randall and Goodbourn, 2008). Through surface-bound receptors and receptors localized in endosomes or in the cytosol, the host cells can respond to approaching pathogens (Figure 3). Toll-like receptors (TLRs) are a type of pattern recognition receptors (PRRs) which recognize specific pathogen-associated molecular patterns (PAMPs) distinguishable from host molecules. The majority of TLRs are transmembrane proteins with a leucine-rich ectodomain and a conserved intracellular domain known as the Toll/interleukin-1 receptor (TIR) domain. These domains trigger a series of mechanisms leading to the synthesis and secretion of cytokines and activation of other host

defense programs that are crucial to the development of innate or adaptative immune responses. So far 13 mammalian TLRs have been identified (Kawai and Akira, 2009). Toll-like receptors bind and become activated by different ligands. TLR3 transduces its signal in response to dsRNA, either viral or synthetic (poly I:C), which are known to be efficient inducers of IFN-α/β (Alexopoulou et al., 2001). Mammalian TLR3 has a wide tissue distribution and is localized either in the endosomal membrane or on the cell surface (Randall and Goodbourn, 2008). This signal transduction is complex and

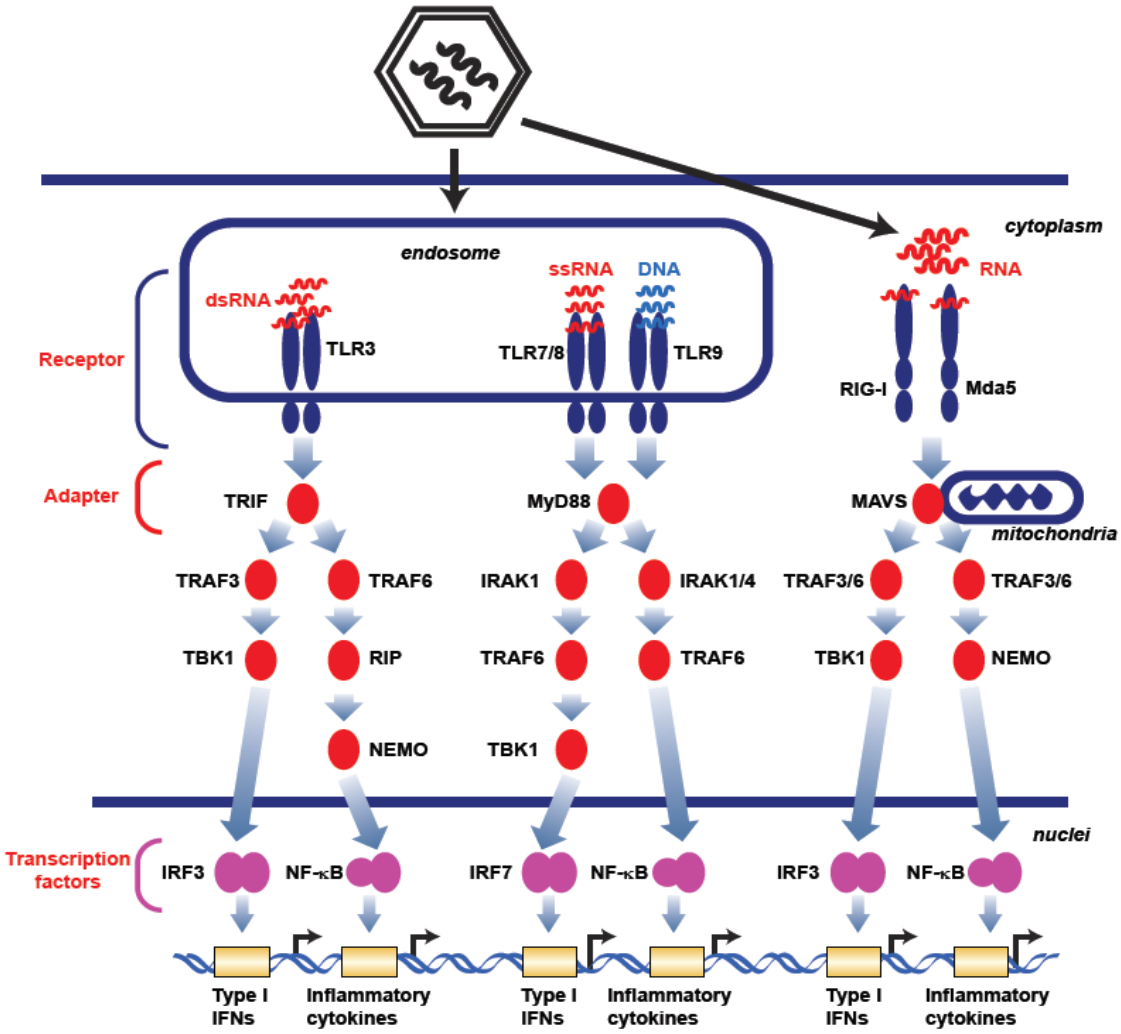


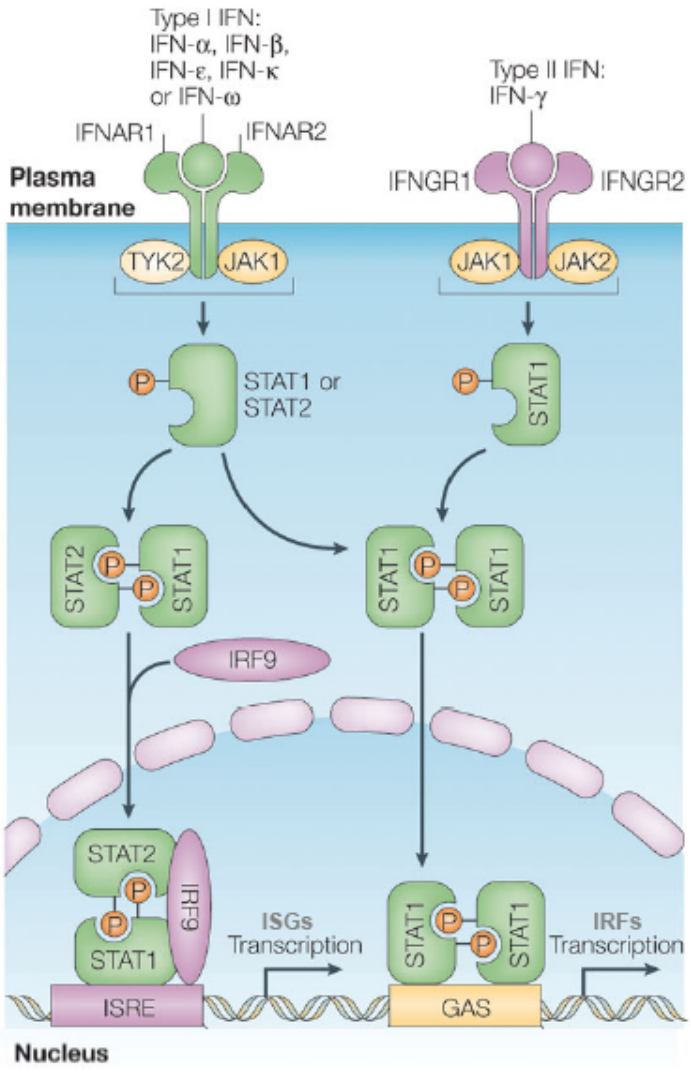
Figure 3. Recognition of viral nucleic acids and induction of interferons. The cytoplasmic receptors RIG- and Mda5 recognize RNA and signals through interaction of their CARD with CARD on the mitochondria-bound adaptor protein MAVS. TLR3, TLR7/8 and TLR9 are endosomal receptors which bind dsRNA, ssRNA and DNA, respectively. TLR3 uses TRIF as an adaptor, while TLR7/8 and TLR9 are MyD88-dependent. Further signaling is cascaded through distinct pathways leading to IRF3 or IRF7 activation and subsequent type I IFN induction, alternatively NF-κB activation and induction of inflammatory cytokines. The figure is modified from (Kawai and Akira, 2008) and (Kawai and Akira, 2006) by R. Cayers.

branches into either an IRF-3 or a NF-κB pathway which both in orchestral cooperation with several other transcription factors eventually lead to activation of the IFN-α/β promoter or induction of pro-inflammatory cytokines, respectively. Upon detection of dsRNA the TIR domain of TLR3 recruits TRIF (TIR-domain-containing adapter-inducing interferon-β) which in turn interacts with either TANK-binding kinase 1 (TBK1) leading to IRF3/IRF7 activation or receptor-interacting protein-1 (RIP1) leading to activation of a large kinase complex consisting of the catalytic subunits IKKα (IκB kinase) and IKKβ plus the NF-κB essential modifier (NEMO). The activated IKK complex phosphorylates the inhibitor of NF-κB (IκB) and targets this inhibitor for degradation by the ubiquitin-proteasome pathway which eventually leads to NF-κB activation (Figure 3). TLR7 is the endosomal receptor of ssRNA, predominantly found in plasmacytoid dendrittic cells (pDC). These cells also harbor TLR9, the receptor of CpG (unmetylated) DNA, in their endosomes, and are dependent on TLRs for IFN induction. The cytoplasmic recognition molecules for viral RNA, melanoma differentiation-associated gene-5 (mda-5) and retinoic acid-inducible gene-I (RIG-I) (Kato et al., 2005; Kawai and Akira, 2005) (described below) are not utilized by pDCs. The TLR7 and TLR9 mechanism of IFN induction follows pathways that are initially dependent on myeloid differentiation factor 88 (MyD88) and interleukin-1 receptor-associated kinases 4 (IRAK4) and IRAK1, but otherwise resemble the pathways initiated by TLR3. Host cells also feature TLR-independent pathways that lead to IFN production. The intracellular dsRNA sensors, mda-5 and RIG-I are widely expressed cytoplasmic RNA helicases which respond to viral nucleic acids present in the cytoplasm after viral replication (Andrejeva et al., 2004; Kawai and Akira, 2008; Yoneyama et al., 2005). RIG-I contains an RNA helicase domain that binds to dsRNA and two amino-terminal caspase activation and recruitment domain (CARD) responsible for protein interaction with the CARD of other proteins required for downstream signaling events (Seth et al., 2005). RIG-I knockout mice are embryonic lethal, however studies using cells derived from these mice show that RIG-I is essential for innate immune responses to several RNA viruses in different cell types (Kato et al., 2005). An adaptor molecule that connects RIG-I sensing of incoming viral RNA to downstream signaling and gene activation has been described by several research groups as a 62 kDa protein and named MAVS (mitochondrial antiviral signaling)/IPS-1 (IFN-β promoter stimulator protein 1)/ VISA (virus-induced signaling adaptor)/Cardif (CARD adaptor inducing IFN-β) (Hiscott et al., 2006b), hereafter termed MAVS. This protein is targeted to the mitochondrial membrane by a C-terminal transmembrane domain, and has an N-terminal CARD that associates with the

CARD regions of RIG-I and mda-5, ultimately leading to the induction of type I IFNs. Silencing of MAVS expression through RNAi abolishes the activation of NF-κB and IRF3 by viruses (and poly I:C), thereby permitting viral replication (except in pDC) (Sun et al., 2006). Mice lacking MAVS are viable and fertile but are severely compromised in immune defense against viral infection. These results provide the *in vivo* evidence that the cytosolic viral signaling pathway through MAVS is specifically required for innate immune responses against viral infection (Sun et al., 2006). Some of the details of this pathway have been elucidated by showing that whereas RNAi of MAVS blocks IFN-induction by RIG-I, RNAi of RIG-I do not inhibit IFN-induction by MAVS, which indicates that MAVS lies downstream of RIG-I. Further experiments has shown that MAVS lies upstream of the kinases that phosphorylate IRF3 (TBK1) and the NF-κB inhibitor, IκB (IKK/NEMO complex) (Seth et al., 2005). This is the first example of a mitochondrial protein with a role in innate immunity. The fact that MAVS localizes to the mithochondrial membrane suggests a link between viral infection, mitochondrial function and development of innate immunity (McWhirter et al., 2005).

JAK/STAT signaling; induction of ISGs

In mammals, the Janus-activated kinase (JAK)/signal transducer and activator of transcription (STAT) pathway is the principal signaling mechanism for a wide array of cytokines and growth factors. Such extracellular signaling polypeptides are recognized by specific transmembrane receptors or receptor complexes on target cells. This recognition leads to a rapid change in the cellular gene expression critical in many biological processes such as developmental regulation, growth control, and homeostasis and anti-inflammatory responses in multicellular organisms. Mechanistically, JAK/STAT signaling is relatively simple, with only a few principal components (Aaronson and Horvath, 2002). Type I IFNs (IFNα/β) induce immune responses through a cell surface, two subunit, transmembrane receptor termed IFNAR1/ IFNAR2 (Figure 4). Ligand binding to this receptor results in activation of the Janus-activated kinase 1 (JAK1) and tyrosine kinase 2 (TYK2) followed by tyrosine phosphorylation of STAT1 and STAT2 (Brierley and Fish, 2002; Garcia-Sastre and Biron, 2006; Platanias, 2005). A heterodimer of STAT1 and STAT2 associates with IRF9, forming the ISGF3 complex which in the nucleus binds to an IFN stimulated response element (ISRE) activating the promoters of IFN-stimulated genes (ISGs). Type II IFN (IFNγ) binds to a different dimeric receptor, the IFNG1/IFNG2 receptor, and induces receptor tyrosine phosphorylation by JAK1 and JAK2 proteins, producing a recruitment site for STAT1 which



in turn is phosphorylated. STAT1 dimerizes and translocates to the nucleus to activate transcription from GAS (gamma-interferon activation site) elements in ISG promoters (Aaronson and Horvath, 2002).

Figure 4. Type I IFNs bind a common receptor composed of the two subunits IFNAR1 and IFNAR2 at the cell surface. The subunits are associated with TYK2 and JAK1, respectively. Type II IFNbinds another receptor which is also composed of two subunits, IFNGR1 and IFNGR2, which are associated with JAK1 and JAK2, respectively. Activation of type I IFN receptor results in tyrosine phosphorylation of STAT2 and STAT1 leading to formation of t STAT1–STAT2–IRF9, known as the ISGF3 complex. These complexes translocate to the nucleus and bind ISREs in DNA to initiate gene transcription. Both type I and type II IFNs also induce the formation of STAT1–STAT1 homodimers that translocate to the nucleus and bind GAS elements that are present in promoter regions. The figure is modified from (Platanias, 2005).

Intercellular signaling is critical for efficient innate immuneresponses, and STAT pathways have been found to be evolutionary conserved from slime molds, worms, flies, and vertebrates but are absent from fungi and plants (Darnell, 1997). In mammals, there are seven STAT proteins that respond to distinct stimuli and induce the transcription of genes that can elicit different physiological outcomes; these are, STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6 (Rogatsky and Ivashkiv, 2006). STAT proteins are localized in the cytoplasm of unstimulated target cells and remain inactive in the absence of receptor-ligand coupling upon which they are rapidly activated through specific binding between STAT Src-homology 2 (SH2) domains and receptor phosphotyrosine residues. These SH2-

phosphotyrosine interactions are highly specific and determine the role of the STATs as transcription factors. All the STAT proteins contain a defined domain like structure (Figure 5). The amino-terminal part of STATs contains a sequence responsible for stabilizing interaction between STAT dimers, thereby forming STAT tetramers. The coiled-coil domain facilitates interactions to other proteins, and the DNA-binding domain (DBD) forms a nuclear localization signal (NLS) upon dimerization. Residues in this domain make direct contact with DNA elements in ISG promoters. A nuclear export signal (NES) is also found in this domain. The carboxy-terminal part of STATs is composed of a SH2 domain featuring a phosphotyrosine binding pocket allowing proteins with phosphorylated tyrosines to bind. Next to the SH2 domain is a transcriptional activation domain (TAD) with a tyrosine phosphorylation site critical for downstream signaling. A conserved phosphoserine site within the TAD mediates interactions between STATs and transcriptional cofactors such as histone acetyl transferases (HATs) and CBP/p300 (Brierley and Fish, 2005).

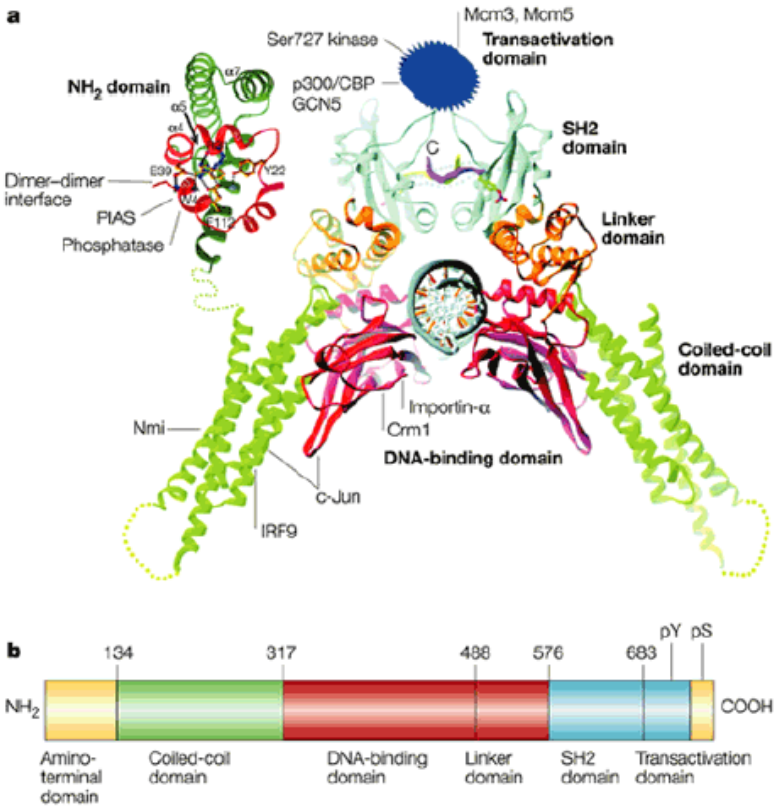


Figure 5. a. Model of the core structure of STAT1 (amino acids ~ 130–712) shows binding of a STAT1 dimer to DNA. **b.** The domain-like structure of STAT1. The figure is from (Levy and Darnell, 2002).

Ways to assassinate "the bad guys"

As a consequence of the IFN signaling trough the JAK/STAT pathway an onset of ISGs leads to an antiviral state in the host cell. Four main effector pathways of IFN-mediated antiviral response has been distinguished: the myxovirus resistance gene (Mx) GTPase pathway, the 2',5'-oligoadenylate-synthetase (OAS)- directed ribonuclease L (RNAse L) pathway, the

protein kinase R (PKR) pathway and the ISG15 ubiquitin-like pathway (Katze et al., 2002; Sadler and Williams, 2008).

Mx proteins are large (~80 kDa) IFN-inducible GTPases with an enzymatic antiviral activity. Mx proteins self-assemble and bind viral nucleocapsids and thereby interfere with intracellular trafficking of virus and the activity of viral polymerases, thus inhibiting replication of many RNA viruses including influenza (Pavlovic et al., 1990) and measles viruses (Schnorr et al., 1993). In humans there are two related Mx proteins (MxA and MxB) which of only MxA appears to have antiviral effect (Haller et al., 1998). The two Mx proteins identified in mice (Mx1 and Mx2) both confer antiviral activity, although at distinct sub-cellular locations and with distinct specificity to various viruses (Jin et al., 2001; Staeheli et al., 1986).

OAS is an IFN-induced, dsRNA-dependent enzyme which converts ATP to 2′,5′-linked oligoadenylates that activate the latent endoribonuclease RNAse L. By degrading ssRNA the RNAse L provides a mechanism of antiviral action, although this pathway also affect cellular RNAs implicating an important role in cell growth, differentiation and apoptosis (Hovanessian, 2007).

PKR is a serine/threonine protein kinase which is dimerized and activated by autophosphorylation upon binding to dsRNA. PKR exhibits antiviral effect by phosphorylating a protein known as eIF-2α, a eukaryotic translation initiation factor. After phosphorylation, eIF-2α forms an inactive complex with eIF-2B, thereby leading to reduced translation initiation and reduced protein synthesis. This inhibits viral replication and normal cell ribosome function, which may lead to killing both the virus and susceptible host cells in "self defense" (Garcia et al., 2006).

One of the most predominantly expressed ISGs is ISG15, a 15 kDa ubiquitin-like protein containing two tandem repeats of an ubiquitin-like (UBL) domain. Accordingly, ISG15 can conjugate to cellular proteins, but do not target them for proteasomal degradation. The C-terminal, encoding conserved hydrophobic patches, is sufficient for activation and transfer of ISG15 to the IFN-inducible enzymes E1 and E2. However, the N-terminal UBL seems to be necessary for E3-mediated conjugation to protein substrates. E1, E2 and E3 proteins sequentially catalyse the conjugation of ISG15 to numerous protein substrates to modulate multiple cellular responses to inhibit virus production. This process (known as ISGylation) is

reversibly regulated by proteases (such as ubiquitin-specific protease 18 (USP18)), which are also induced by IFNs (Sadler and Williams, 2008).

In addition, the IFN-inducible RNA-specific adenosine deaminase ADAR1 can have an antiviral effect (George and Samuel, 1999; Patterson et al., 1995). Viral RNAs are subjected to A-to-I editing resulting in transcripts that differ from their template. Thus, such RNA modification has the potential to alter the protein-coding capacity of the edited transcript. The A-to-I transitions also destabilize the dsRNA helix by disrupting base pairing. A cytoplasmic endonuclease which specifically cleaves hyper-edited inosine-containing dsRNA (Scadden and Smith, 2001), may be an important factor affecting the stability of I-containing RNAs. ADAR was first identified as a dsRNA-unwinding enzyme in *Xenopus* oocytes (Bass and Weintraub, 1988). The ~150 kDa human ADAR1 deaminase was isolated in a screen for IFN-regulated cDNAs (Patterson and Samuel, 1995) and has a domain-like structure consisting of 3 dsRNA-binding domains, two Z-DNA-binding domains in addition to a catalytic domain (Lai et al., 1995; Liu et al., 1997). ADAR1 transcription levels are induced by both IFN-α and IFNγ (Patterson et al., 1995). There also exist a smaller (110-kDa), non-inducible form of ADAR1 which localizes to the nucleus in human cells (Patterson and Samuel, 1995). Additionally there is an ADAR2, not inducible by IFN, present in human cells (Patterson and Samuel, 1995). A proviral effect of ADAR1 has also been reported (Nie et al., 2007). Replication of vesicular stomatitis virus (VSV) is enhanced by ADAR1 through a mechanism independent of dsRNA editing. The ADAR1 interacts with PKR, inhibits its kinase activity thereby suppressing eIF-2α phosphorylation. Consistent with the inhibitory effect on PKR activation, ADAR1 increases VSV infection in PKR+/+ mouse embryonic fibroblasts; however, no significant effect was found in PKR-/- cells. This proviral effect of ADAR1 requires the N-terminal domains but does not require the deaminase domain (Nie et al., 2007).

Finally, many IFN-pathway signaling proteins are themselves ISGs, thus providing a feedback loop that amplifies IFN responses; examples are IRF7, RIG-I, Mda-5 and STAT1. ISGs are diverse in their biological functions and numerous genes are known to be highly induced at transcriptional levels, however many are still poorly characterized functionally. With further functional studies, some additional genes of the about 300 genes that are induced following treatment with IFN and identified by oligonucleotide microarray studies, could prove to be critical mediators of antiviral actions (Maher et al., 2007; Samuel, 2001).

Viral "under cover operations"

In general it seems difficult for a virus to overcome the powerful IFN-induced antiviral mechanisms the host cells possess. Anyhow, viral infections and replication occur, enabled through a variety of mechanisms that have evolved in viruses to evade host defense. It is shown that many viruses dedicate parts of their genome to encode gene products able to counteract components of the IFN pathways (Garcia-Sastre and Biron, 2006). The strategies could be either to antagonize IFN induction, IFN signaling, expression or action of ISGs (Biron, 2001). Influenza A/B (*Orthomyxoviridae*) NS1 protein (Wang et al., 2000), vaccinia virus (*Poxviridae*) E3L protein (Chang et al., 1992) and the NSP3 protein of rotavirus (*Reoviridae*) (Langland et al., 1994) are examples of well known IFN-antagonists of viral origin that all block initiation of IFN production at the transcriptional level. Other viruses encode proteins that block IFN signaling. Adenovirus (*Adenoviridae*) protein E1A decreases levels of STAT1 and IRF9 and inhibits the binding of co-activator CBP (CREB binding protein) by directly interacting with STAT1 (Leonard and Sen, 1996; Look et al., 1998). Simian virus 5 (SV5**,** *Paramyxoviridae*) also have a protein (V) that blocks IFN-α/β and IFNγ by targeting STAT1 for proteasome-mediated degradation (Didcock et al., 1999). A common strategy developed among many viruses is down-regulation of PKR activity to avoid jeopardizing their own survival and viral replication. By binding and sequestering dsRNA, viral proteins can deprive PKR of its activator and thereby inhibit its kinase activity (Gale and Katze, 1998). Poxviruses (*Poxviridae*) possess multiple strategies of immune evasion; they can secrete decoy IFN receptors to prevent initiation of IFN signaling, a viral phosphatase dephosphorylates STAT1, and the myxoma virus (MV) is able to block tyrosine phosphorylation of TYK2 (Wang et al., 2009). The nonstructural protein NS5A of Hepatitis C virus (HCV) is a promiscuous protein with multiple interaction partners among own viral proteins and cellular proteins. It shows binding properties to PKR and inhibits binding to, and activation by dsRNA. It also leads to up-regulation of pro-inflammatory cytokine interleukin 8 (IL-8). Both properties result in inhibited IFN activity. Through its interactions NS5A also modulates mitogen-activated protein kinase (MAPK) signaling pathways and inhibits antiviral actions of IFNs (Macdonald and Harris, 2004). Lower expression levels of type I IFN, STAT1 and IRF9, in addition to reduced DNA-binding activity of the STAT/IRF9 complex to the ISG promoter upon HCV infection, indicate additional inhibition of the JAK/STAT signaling pathway (Blindenbacher et al., 2003). This impairment of JAK/STAT signaling is probably caused by inhibition of the extracellular signal-regulated kinase (ERK) MAPK

through an interaction between NS5A and the adaptor protein Grb2 (Tan et al., 1999). Furthermore, the HCV NS3/4A protease specifically cleaves MAVS as part of its immune-evasion strategy (Hiscott et al., 2006a). These studies highlight the diversity of survival strategies among viruses.

What about fish?

Fish are now known to possess many of the ligands, receptors, and signaling adaptor molecules necessary for antiviral responses, and knowledge about the fish immune system is emerging, although some key molecules known in mammalian systems are still not identified or characterized in fish.

Virus recognition / IFN-initiation in fish

Signaling pathways for induction of IFN in teleost fish are likely to be similar to mammalian pathways as fish homologs of receptors and downstream signaling factors have been identified (Purcell et al., 2006; Stein et al., 2007). Receptors for viral nuclear acids such as RIG-I and mda-5 (recognition of dsRNA) are strongly expressed in salmon head kidney leukocytes, whereas TLR7/8 (recognition of ssRNA) and TLR3 (recognition of dsRNA) are expressed at much lower levels (Sun et al., 2009), indicating that RIG-I and mda-5 might be the major viral sensors in salmon. Recently RIG-I along with MAVS was cloned from several fish species including Atlantic salmon (Biacchesi et al., 2009). Additionally, sequences related to MAVS has been identified even in the lowest vertebrates, chondrichthyes (cartilaginous fish) and agnatha (jawless vertebrates) indicating the importance of this molecule in evolution. Like the mammalian MAVS, salmon MAVS is a transmembrane protein located at the mitochondria. Overexpression of MAVS induces antiviral immunity against several viruses (VHSV, IHNV and SVCV) and is dependent on the CARD and transmembrane domains (Biacchesi et al., 2009). The spleen is shown to be the salmon organ expressing the highest levels of TLR8, but TLR8 is also present at low levels in head kidney, kidney and gills (Skjæveland et al., 2009). Pufferfish and zebrafish have a broader distribution of TLR8 in their organs, whereas low TLR8 expression is found in the spleen from these species (Jault et al., 2004; Oshiumi et al., 2003). In the Atlantic salmon cell-line TO TLR7/8 is weakly expressed, but is inducible by both type I and type II IFN (Skjæveland et al., 2009; Sun et al., 2009). Distinct recognition patterns may apply in different cell types. TLR3 is cloned from rainbow trout and displays a genomic organization similar to mammalian and other fish TLR3 genes. It responds with increased levels of gene expression upon stimulation

with poly I:C and challenge with IHNV (Rodriguez et al., 2005). The discovery of a dsRNA receptor, named TLR22, shows that fish have a dual dsRNA recognition system. TLR22 is is unique for lower vertebrates and is localized to the cellular membrane, recognizing extracellular nucleic acids (Matsuo et al., 2008). Despite extensive searching (Roach et al., 2005) this subfamily of TLRs have not so far been identified in mammals.

IFN genes in fish

Currently, only type I and II IFNs have been identified in fish. The two types of IFN are not structurally related, and are classified on basis of their receptor recognition and mechanisms of gene activation. All fish IFN genes identified contain four introns (except Japanese medaka IFN with three introns), a gene structure resembling mammalian IFNλ (type III IFN), while their protein sequences are more similar to mammalian IFNα/β. Hence, a certain disagreement on how to classify the teleost IFNs has become evident as the high degree of sequence divergence from mammalian IFN has made grouping equivocal (Levraud et al., 2007; Robertsen, 2006; Sun et al., 2009).

IFNs, which functionally correspond to mammalian type I IFNs, have been cloned from a number of fish species, including Atlantic salmon, rainbow trout, channel catfish, pufferfish, stickleback, zebrafish, Japanese medaka and recently, sea bass (Casani et al., 2009; NCBI, 2009; Robertsen, 2006). Lately fish type I IFNs have been further classified into two groups based on their primary protein sequences. In rainbow trout three type I IFNs have been isolated. Like most mammalian type I IFNs (except β and ε), recombinant rtIFN1 and rtIFN2 both contain two cystein residues. They both up-regulate expression of Mx and inhibit VHSV replication in RTG-2 cells. These are placed in group I. In contrast, recombinant rtIFN3 belonging to group II was found to be a poor inducer of Mx and antiviral activity. The three rtIFNs also showed differential expression in cells and tissues (Zou et al., 2007), suggesting distinct functions of the three subtypes in the immune system of fish. A similar sequence-based grouping has been designated in zebrafish. Whereas group I contains one gene with a powerful, but slow rate, antiviral and pro-inflammatory induction, group II consists of two genes unable to induce inflammatory response, but rather induce a rapid and transient expression of antiviral genes. Thus, these two groups of IFN genes have complementing roles (Lopez-Munoz et al., 2009). Several type I IFNs have been characterized in Atlantic salmon (Robertsen et al., 2003; Sun et al., 2009). Elleven genes encode IFNs that can be categorized into three subtypes; IFNa (two genes, IFNa1/IFNa2), IFNb (four genes) and IFNc (five genes). IFNa posses an NF-κB site in their promoters and are strongly up-regulated by poly I:C,

suggesting that salmon IFNa1/IFNa2 are induced through similar pathways as human IFN-β. IFNb genes are suggested to be induced through a pathway similar to mammalian IFN-α, whereas IFNc genes showed expression patterns different from both IFNa and IFNb (Sun et al., 2009).

IFNγ, mainly produced by $CD4^+$ T helper 1 (Th1) lymphocytes and NK cells in response to MHC-presented antigens, is regarded as one of the hallmarks of the Th1 immune response in birds and mammals. Whether lower vertebrates such as fish have developed the equivalent of a Th1 immune system has been questionable until the IFNγ gene was identified in rainbow trout (Zou et al., 2005), two species of puffer fish (Igawa et al., 2006; Zou et al., 2004), zebrafish (Igawa et al., 2006), channel catfish (Milev-Milovanovic et al., 2006) and in goldfish (Grayfer and Belosevic, 2009). Some of its biological activities was characterized in fish for the first time by Zou et al. (2005) who demonstrated that trout IFNγ was functionally similar to its mammalian counterpart, and that at least some of the intracellular signaling pathways are conserved. In addition fish IFNγ has proven to induce antiviral ISGs such as Mx (Jørgensen et al., 2007; Martin et al., 2007b) and also TLR8 and TLR9 in salmon (Skjæveland et al., 2009; Skjæveland et al., 2008). Several fish species have two evolutionary conserved IFN-gamma genes possibly as a consequence of a duplication event in teleosts after the tetrapod-fish split (Savan et al., 2009). The presence of key elements like types I and II IFN and NK cells witness of a fish immune system not unlike the one found in mammalian. Revealing whether salmonids and other fish species possess specialized high IFN producing cells such as pDCs or if these cells appeared later in evolution will shed new light on the evolutionary process of the immune system.

IFN receptors in fish

A two subunit receptor (zCRFB1 and zCRFB5) of the class II helical receptor family has been identified for zebrafish virus-induced IFN (zIFN) (Levraud et al., 2007). The authors of this paper have classified the virus-induced IFN and its receptor as type III IFN, based on the organization of the *zIFN* gene (four introns) and the protein structure of the identified receptor components. These authors have previously also described this family of receptors (CRFB 1-9) in *Tetraodon nigroviridis* (green spotted puffer) (Levraud et al., 2007; Lutfalla et al., 2003). Other authors claim that these IFNs characterized in zebrafish and pufferfish resembles the type I IFNs characterized from other fish species and hence these receptors must be appointed type I IFN receptors (Børre Robertsen, personal communication). Recently, two putative

transmembrane receptor chains for fish IFNγ were cloned from rainbow trout (Gao et al., 2009). Characterization by conserved domain analyses, phylogeny, expression analyses and ligand binding analyses all suggest that these novel molecules resemble the mammalian IFNγ receptors.

IFN signaling in fish

Although little is known about the factors that contribute to IFN signaling in fish, some proteins involved in the signaling pathway have been cloned. TYK2 and JAK1 have been cloned from pufferfish (*Tetraodon fluviatilis*) (Leu et al., 1998; Leu et al., 2000), and STAT1 has been cloned from zebrafish (Oates et al., 1999), crucian carp (Zhang and Gui, 2004), rainbow trout (GeneBank accession nos: U60331, U60332) and Atlantic salmon (own unpublished data; (Collet et al., 2008)). Functional analyses of zebrafish STAT1 protein shows that it rescues interferon-signaling in a STAT1-deficient human cell line (Oates et al., 1999) This indicates that cytokine signaling mechanisms are likely to be conserved between fish and tetrapods. Recently also a STAT2 protein was identified in Atlantic salmon (Collet et al., 2009).

Fish Mx promoter

The rainbow trout Mx1 promoter has been cloned and characterized and was shown to contain a TATA box at −29 to −25, a 13 nucleotide ISRE between −101 and −89, and a Sp1 binding site at −382 to −374. The ISRE element with the sequence TGAAAGTGAAACA had high homology to ISRE elements found in mammalian IFN-induced genes (Collet and Secombes, 2001). The rainbow trout Mx promoter has also been shown to respond to poly I:C (Collet and Secombes, 2001) as well as both salmon IFN-α/β and trout IFNγ in a dose-dependent manner, while there was no response to TNF-α and IL-1β (Johansen et al., 2004). A construct featuring the Mx promoter in front of a luciferase reporter gene has been a useful tool for studying regulation and induction of IFN responses in teleost fish (Johansen et al., 2004). A salmon cell line with this construct integrated in the genome has been established as an even more utile tool for these studies (Jørgensen et al 2007). Also Japanese flounder Mx is cloned and similar to rainbow trout its promoter contains a TATA box (-24 to -30, whereas it has two ISREs (-69 to -80 and -508 to -521) and two Sp1 sites (-563 to -572 and -994 to -1003) (Ooi et al., 2006). Mx expression is increased more than 10-fold after stimulation by poly I:C. Deletion mutational analyses has revealed that the ISRE closest to the transcription start site is crucial for promoter activity. The distal ISRE is not able to induce promoter activity by itself,

but contributes to maximal activity in co-presence with the proximal ISRE. Both ISREs bind transcription factors. Induction of luciferase by poly I:C is inhibited by a PKR inhibitor, in a dose-dependent manner, suggesting that PKR may be required as a signal transducer for type I IFN signaling in fish (Ooi et al., 2006).

Fish ISGs

Until recently, few ISGs had been identified in fish. A study using Atlantic salmon cDNA microarray showed up-regulation of 47 genes in an Atlantic salmon cell line (SHK-1) stimulated by recombinant trout IFN1 and by type II IFN, 72 partially overlapping genes were upregulated (Martin et al., 2007b). Mx is still the best characterized among them. The Mx gene has been cloned from many different species including Atlantic salmon, rainbow trout, Atlantic halibut, Japanese flounder, channel catfish, gilthead sea bream and zebrafish. Expression studies of Mx has shown that transcription and protein synthesis is inducible by IFN, poly I:C and viral infections (Robertsen, 2006). The protein is expressed in the nucleus as well as in the cytoplasm and has been proven to have antiviral effect against IPNV in salmon (Larsen et al., 2004), the IPNV related sole aquabirnavirus in Senegalese sole (Fernandez-Trujillo et al., 2008), IHNV in brown trout (Saint-Jean and Pérez-Prieto, 2007), a nodavirus in grouper (Lin et al., 2006) and two rhabdoviruses in Japanese flounder (Caipang et al., 2003). The ubiquitin-like protein ISG15 is also identified in fish species like Atlantic salmon (Røkenes et al., 2007), Atlantic cod (Seppola et al., 2007), crucian carp (Zhang et al., 2007) and black rockfish (Baeck et al., 2008). Like Mx, this gene is highly induced by IFN, poly I:C and viral infection. Among the less chatacterized proteins are 10 virus-induced genes (vigs) identified in rainbow trout which are also found to be induced by IFN-like activity in trout leukocytes (Babiuk et al., 2000; Boudinot et al., 1999; Boudinot et al., 2001; O'Farrell et al., 2002). *vig-3* has a sequence very similar to Atlantic salmon ISG15. *vig-4* is homologous to the ISG56/ISG58 family of mammalian proteins, which are proteins that interfere with protein synthesis. *vig-7* and *vig-8* are homologous to CXC chemokines. The chemokine CXCL10 and two proteins of unknown function, ISG12 and GIG2, have been identified as IFN-induced proteins in Atlantic salmon (Martin et al., 2007a; Robertsen, 2008). PKR-like eIF2α kinases called PKZ due to their Z-DNA-binding capacity have been discovered in fish. PKZ is structurally similar to mammalian PKR with 11 conserved kinase subdomains in the C-terminal catalytic domain whereas the N-terminal domain contains two Z-DNA-binding domains instead of the two dsRNA binding motifs seen in PKR (Bergan et al., 2008; Hu et al., 2004; Rothenburg et al., 2005). PKZ is up-regulated by viral infection and has a possible

capability of preventing protein synthesis, although the ability to inhibit virus replication is unknown (Rothenburg et al., 2005; Zhu et al., 2008). Additionally, a cDNA encoding PKR protein has recently been isolated from Japanese flounder and found to interact with, and be responsible for phosphorylation of eIF2α and thereby confer antiviral activity against *Scophthalmus maximus* rhabdovirus (SMRV) (Zhu et al., 2008). Also fish homologs of the A-to-I RNA editase ADAR have been identified in two species of pufferfish in addition to zebrafish (Slavov et al., 2000). The gene structure and organization are highly conserved; although the different RNA-binding domains (RBD1/2/3) have different levels of conservation, which probably reflect the differences in editase substrate specificity and/or substrate sequence conservation (Slavov et al., 2000). Although nine IRF members are found in mammals only fish homologues of IRF1, IRF2, IRF3 and IRF7 have been identified. These are all trancriptionally up-regulated by viruses and dsRNA (poly I:C) (Caipang et al., 2005; Collet and Secombes, 2002; Holland et al., 2008; Ooi et al., 2006). Rainbow trout IRF3 genes are transcriptionally modulated by the same range of ligands as IRF7, which is distinct from mammals, where IRF3 transcription is not influenced by viral infection nor IFN treatment (Holland et al., 2008). Only IRF1 has been functionally assessed in fish through the examination of nuclear translocation events and the ability of over-expressed IRF1 to induce an antiviral state in carp EPC cells (Caipang et al., 2005; Ooi et al., 2006).

IFN antagonism in fish

The recent literature is rich with descriptions of viral interference with antiviral signaling in mammals (Haller et al., 2006; Randall and Goodbourn, 2008; Unterholzner and Bowie, 2008), still little is known about the ability of fish viruses to antagonize the host immune responses. One recent example, though, is the infectious salmon anemia virus (ISAV), which is closely related to influenza A. An ORF on segment 7 of ISAV encodes a protein (s7ORF1) which has shown abilities to down-regulate the action of IFN rather than the production (McBeath et al., 2006). However, this is contradicted by García-Rosado et al. (2008), where the s7ORF1 protein and a protein encoded by segment 8 (s8ORF2) both are, independent of each other, shown to inhibit the type I IFN promoter activation. Another example of immune evasion is from betanodavirus, a bipartite, positive-sense RNA virus, which is the causative agent of viral nervous necrosis in marine fish. The B2 protein encoded by this virus is a sequence-independent dsRNA binding protein. By coating the viral dsRNA it prevents Dicer enzyme from generating small interfering RNA (siRNA). dsRNA-sequestration also enables this protein to interact with the dsRNA binding domain of ADAR1 and thereby block RNA

editing. Despite this activity, B2 is unable to block the induction of the dsRNA-dependent IFN response (Fenner et al., 2006). There are also indications that IPNV possess strategies to circumvent host immunity. Infection with IPNV in RTG-P1 cells stimulated with IFN is found to significantly suppress Mx transcription compared to uninfected, IFN-stimulated cells (Collet et al., 2007).

Aims of study

Major loss in farming of Atlantic salmon is caused by IPNV. The existing vaccines do not give satisfactory protection. In order to optimize efficacy of vaccines, the mechanisms mediating protection against IPNV in salmonids need to be elucidated. Knowledge about IPNV interactions with the host's antiviral immune defence can contribute to development of efficient prophylactic strategies. The aims of this project were to:

- Apply new molecular techniques such as the yeast two-hybrid system and co-immunoprecipitation to study protein-protein interactions among IPN viral proteins.
- Study protein-protein interactions between IPN viral proteins and host proteins.
- Study the protective ability of recombinant IFN-α1 against IPNV infection.
- Study the IFN-antagonistic properties of IPNV and elucidate candidate molecules responsible for these properties.
- Functional studies of novel Atlantic salmon molecules involved in the JAK/STAT signaling pathway.

Summary of papers

Paper 1. VP3, a structural protein of the infectious pancreatic necrosis virus, interacts with the RNA-dependent RNA polymerase VP1 and with double-stranded RNA.

Torunn Pedersen, Astrid Skjesol and Jorunn B. Jørgensen

In paper 1 we focus on the interaction properties of VP3, the suggested key organizer of particle assembly in birnaviruses. By applying the yeast two-hybrid system in combination with co-immunoprecipitation, VP3 was proven to bind to VP1 and to self-associate strongly. In addition, VP3 was shown to specifically bind to dsRNA in a sequence independent manner by *in vitro* pull-down experiments. The binding between VP3 and VP1 was not dependent on the presence of dsRNA. Deletion analyses mapped the VP3 self-interaction domain within the 101 N-terminal amino acids and the VP1-interaction domain within the 62 C-terminal amino acids of VP3. The C-terminal end was also crucial, but not sufficient for the dsRNA binding capacity of VP3. For VP1, the 90 C-terminal amino acids was the only dispensable part for maintaining the VP3-binding ability. Kinetic analysis revealed the presence of VP1-VP3 complexes prior to the formation of mature virions in IPNV-infected CHSE-214 cells, which indicates a role in promoting the assembly process.

Paper 2. The interplay between infectious pancreatic necrosis virus (IPNV) and the IFN system: IFN signaling is inhibited by IPNV infection

Astrid Skjesol, Toril Aamo, Marit Nøst Hegseth, Børre Robertsen and Jorunn B. Jørgensen

Indications of IPNV being able to evade or counteract innate host defense come from its lack of ability to induce strong type I interferon (IFN) responses in cell culture. We show in paper 2 that administration of salmon rIFN-α1 to cells prior to IPNV infection halts the viral protein synthesis and prevents processing of pVP2 into mature VP2. Furthermore, compared to pretreatment with IFN-α1 the antiviral state in cells infected with IPNV prior to IFN treatment, was antagonized by IPNV. This was shown by higher viral titers, faster viral protein synthesis and also by reduced Mx expression. The longer headstart the virus gets, the more prominent is the weakening of IFN signaling. IPNV VP4 and VP5 inhibit IFN-induced expression from the Mx promoter, indicating that these proteins contribute to the antagonistic effect.

Paper 3. Structural and functional studies of the STAT1 gene from Atlantic salmon

Astrid Skjesol, Tom Hansen, Cheng-Yin Shi, Hanna Leena Thim and Jorunn B. Jørgensen

In paper 3 we identified a salmon STAT1 homologue with a domain-like structure and functional motifs similar to STAT1 in higher vertebrates. These include an amino-terminus that stabilizes interaction between STAT dimers in a promoter-binding situation, a coiled coil domain facilitating interactions to other proteins, a central DNA-binding domain, a SH2 domain responsible for dimerization of phosphorylated STATs and conserved phosphorylation sites within the carboxy terminus. The latter is also the transcriptional activation domain. The salmon STAT1 was shown to be ubiquitously expressed in the cytoplasm of various cells. It was tyrosine phosphorylated in response to recombinant type I and type II IFNs both in primary leukocytes and in a salmon cell-line. Treatment with (type II) IFN also led to nuclear translocation of STAT1 within one hour. The ability of salmon STAT1 to dimerize was also shown in this paper.

General discussion

One of the most important functions of the IFN system is to inhibit viral infections. The delicate balance between the antiviral mechanisms of the host cells and the viruses' counteracting mechanisms has been the subject for this study. Characterization of interactions between IPNV proteins was our first approach to better understand this interplay from the viral perspective (paper 1). We discovered that the structural protein VP3 was able to bind to dsRNA offering a possible explanation for the inability of IPNV to induce IFN and ISGs in certain cell-lines. If the viral dsRNA is being chaperoned or masked by VP3, and thereby stays hidden from the cell's dsRNA sensors, the antiviral induction might be avoided. Such a role for VP3 has yet to be proven. IPNV seemingly exerts several approaches to convey the IFN system, as this virus is able to counteract type I IFN even when IFN is supplemented to cells during viral infection. Although, administering of IFN to cells 24 h prior to IPNV infection settles a strong antiviral state which the virus is unable to overcome. In this situation, the induction and Mx-expression is high, the viral titers low, and interestingly, the processing of VP2 is delayed or inhibited. When the virus gets a headstart on IFN signaling though, induction of Mx is lowered, viral titers increased and VP2 processing sustained. Analysis of the individual IPNV protein's ability to antagonize induction of the Mx-promoter when stimulated with IFN revealed that VP4 and VP5 were the two most presumtive candidates causing this effect (paper 2). The JAK/STAT signaling pathway is the signature IFN signaling pathway downstream of IFN production. The presumed targets for the antagonistic effect possessed by VP4 and VP5 is most likely molecules along this route. In fish, many of the players involved in signaling downstream of IFN is not yet characterized. In this work, we have characterized a major player in this pathway; STAT1. By utilizing a STAT1 specific antibody we showed that salmon STAT1 was tyrosine phosphorylated and translocated into the nucleus (within 1 h) upon IFN stimulation in certain salmon cell types (paper 3). The expression level of STAT1 mRNA in various tissues was examined in addition to STAT1 mRNA and protein expression upon IFN and viral stimuli in various cell types. The STAT1 reported by us was slightly different from two other salmon STAT1s recorded in GenBank, although they all shared the domain-like structure typical of the STAT proteins. Despite the evolutionary conservation and apparent similar functionality of the IFN system among the vertebrates, differences occur, and further characterization is needed in fish to fully understand the interplay between pathogens and host cells. One interesting differences between mammals and piscine species is the existence of a surface RNA recognition system,

namely TLR22. Pufferfish TLR22 expression responds to double-strand RNA similarly to how TLR3 responds in humans (Matsuo et al., 2008). This respons is also similar to the TLR3 response in fish. The question is why teleosts have doubled their inventory of dsRNA recognition receptors.

The results are discussed in detail in the respective papers. In the following sections some selected topics, updates and general aspects of this work will be addressed.

The significance of the protein-protein interactions in IPNV

New approaches and techniques within the field of fish immunology, such as the yeast two-hybrid (Y2H) system combined with co-immunoprecipitations have provided extended knowledge about each individual protein that constitutes the IPN virion and how they interact with each other. This might contribute to our understanding of the role each protein has in the replicative cycle of the virus and defining specific factors required for infectious virus production and persistence. Our studies (in paper 1) revealed that VP3 interacts with itself through an N-terminal domain. The self-interaction enables the scaffolding of the capsomer, even without a detectable direct interaction with the outer capsid protein VP2. Through its C-terminal VP3 interacts with the RdRp VP1. This interaction is dependent on a full-length VP1 protein, and the interaction domain on VP3 has recently, by a peptide-array been more closely mapped to the 10 C-terminal amino acids (results not shown). This complex formation is RNA-independent and takes place before viral particles appear. Thus it seems to play a role in viral assembly. Additionally, VP3 binds to dsRNA in a sequence-independent manner in the absence of other factors and confers a status as a multipotent protein within the virion. This was detected applaying an *in vitro* translation and dsRNA-binding assay. As expected, we also found that the outer capsid protein VP2 was able to self-interact (results not shown). To further evaluate the function of each IPNV protein and the significance of their interactions, reverse genetics would be a valuable and necessary tool. By reverse genetics also the importance of molecular differences between isolates with high and low virulence can be elucidated. This technique has been applied for IPNV and other birnaviruses by other research groups (Da Costa et al., 2002; Yao and Vakharia, 1998), although attempts to establish this technique for IPNV have not so far been successful in our lab. Resolving the structure of proteins by X-ray crystallography might also reveal useful information about the individual IPNV proteins and their putative functions. New crystallography research on IBDV has revealed that the interaction of the VP3 C-terminal with VP1 promotes conformational change, making the polymerase active site available (Garriga et al., 2007). Thus, VP3 acts as a

transcriptional activator consistent with the structural features revealed by crystallization of the central region of VP3 (Casañas et al., 2008). The structure of the VP4 Ser/Lys proteases from IPNV along with other birnaviruses, such as the blotched snakehead viris (BSNV) (Feldman et al., 2006) have been revealed. This has provided insights into the catalytic mechanism and substrate recognition of these types of proteases, making VP4 protease a potential target for antiviral therapy. Although IPNV and BSNV VP4 share only about 19% sequence identity, their overall structure and probably also their function is much conserved.

The role of IPNV proteins interactions with host cells

Probably the most important, but also the most challenging problem in IPNV biology is to define the specific factors in the host environment that is affected by IPNV infection. There are several possible approaches to target these factors. The yeast two-hybrid system is a tool which can bee applied in screening for host molecules interacting with viral proteins, using a whole library approach. Other methods such as co-IP using virus protein antibodies and subsequent identification of interacting proteins by protein sequencing (MALDI/TOF MS) can be utilized. A virus overlay protein binding assay (VOPBA) has been applied by Ørpetveit et al. (2008) in their search for host proteins binding to IPNV proteins. From a membrane fraction of cellular proteins, a band of high molecular weight (~220 kDa), but with unidentified sequence, was shown to interact with IPNV. This protein was detected by a VP3 antibody (in addition to anti-IPNV) which is surprising since VP3 is thought to be a protein contained within the particle. This is however debatable, as some authors claim that a portion of VP3 is displayed on the virion surface (Caswell-Reno et al., 1986; Tarrab et al., 1993). Similarly a 250 kDa unidentified cellular protein from three fish cell-lines has been proposed to be the virus receptor for the closely related marine aquabirnaviris (MABV) based on interaction in a VOPBA (Imajoh et al., 2003). We show in paper 2 that processing of the premature pVP2 into the mature VP2 is stalled in the prescence of IFN prior to infection. In IBDV, processing of pVP2 (residues 1 to 512) is studied further by a reverse genetics system. Processing of pVP2 generated VP2 and four small peptides (6, 6, 10 and 45 amino acids in length, respectively). At least three of these peptides are associated with the viral particles. Mutants lacking the two smallest peptides are viable, although the virus growth is affected. In contrast, deletions of either of the two largest peptides do not allow virus recovery. Substitution analyzes pinpoints the essential residues to be mainly at the cleavage sites (Da Costa et al., 2002) .

Immune-regulatory responses to IPNV

Viral infection certainly triggers induction of IFNs and ISGs in vertebrates due to the large number of immune cells and the many viral sensors in host cells. IPNV induces both IFN and Mx in Atlantic salmon *in vivo* (McBeath et al., 2007). However, induction is cell-type specific, as IPNV induces neither IFN nor Mx or ISG15 during infection of TO cells or CHSE-214 cells (Jensen and Robertsen, 2002; Røkenes et al., 2007), while IFN-transcripts are induced in Atlantic salmon macrophages and RTG-2 cells upon IPNV infection (Collet et al., 2007). Additionally, IFN-stimulated genes such as IRF1 and IRF2 are up-regulated during IPNV infection in RTG-2 cells (Collet et al., 2007). Ingerslev et al. (2009) have shown that in addition to type I and II IFN and Mx, IL-10 is highly induced in Atlantic salmon lymphoid organs after challenge with IPNV. In mammals, IL-10 is known as a Th2 cytokine, which inhibits synthesis of cytokines in an immunosuppressive manner. However, piscine IL-10 is suggested to function as an inflammatory cytokine. IL-10 is also known as an inhibitor of IFNγ in mouse (Inoue et al., 2005). Another marker for innate immune response, the proinflammatory cytokine IL-1β is weakly induced by IPNV infection. The T-cell receptor-α (TCR-α), its associated molecule CD8-α and the major histocompatibility complex class-I and II molecules (MHC-I and II) are markers of T-cell immunity and all of these except MHCII, are moderately induced by IPNV. Also a marker for humoral immunity, the gene coding for membrane bound immunoglobulin isotype M (mIgM) is moderately upregulated (Ingerslev et al., 2009). IPNV infection induces salmon Annexin 1, an anti-apoptotic gene (Hwang et al., 2007), possibly a viral strategy to promote successful replication. Knocking down Annexin 1 expression by siRNA is not of significance for the expression levels of IPNV proteins at early stages of infection (until 10 h p.i.) although titers are reduced by 75% and apoptosis increased. No direct interaction between Annexin 1 and viral proteins is reported. Preliminary results from a microarray showing gene expression in head kidney from fish challenged with a virulent field isolate of IPNV and a nonvirulent field isolate compared to uninfected fish shows slight up-regulation of several genes (between 1.1 and 2.3 fold) in the most virulent strain at 13 days p.i. (own unpublished results). Only few of these up-regulated genes are known to be involved in IFN signaling, supporting our previous results showing that IPNV does not induce IFN efficiently and/or IFN signaling is inhibited. A closer examination of gene expression (by qPCR) of some genes of interest from the same material, showed a 8-fold induction of STAT1 and a 3 fold up-regulation of TLR8 with the most virulent strain, whereas the TLR22 was not induced (own unpublished results).

Vaccine development

Some effort has recently been made to improve the efficacy of vaccines against IPNV. New reports show that alternative ways of expressing the viral capsid protein VP2 give distict antibody responses. Small (~20 nm) subviral particles composed solely of VP2 are formed by expression of the VP2 gene in yeast. These particles induce anti-IPNV antibodies in rainbow trout vaccinated either by injection of purified VP2-subviral particles or by feeding recombinant yeast expressing VP2. A challenge study of vaccinated trout with a heterologous IPNV strain shows that both injection and orally vaccinated fish have lower IPNV loads than the non-vaccinated fish (Allnutt et al., 2007). In a different study, VP2 isolated and purified from IPNV virions gives a more responsive antibody when injected in rabbits than a recombinant VP2 expressed from *E.coli*. Both antisera react equally well with authentic and recombinant VP2 in Western blots and ELISAs. However, only the antibodies made against authentic VP2 neutralize IPNV infection in CHSE-214 cells and aggregate virions, indicating that a posttranslational modification of the virus protein such as carboxylation is required (Fridholm et al., 2007). Initial steps have recently been taken in the development of DNA-vaccines against IPNV. Experimental vaccination of Atlantic salmon with a plasmid that express the entire large ORF of IPNV Segment A gives a high level of protection, whereas other plasmids expressing only VP2 give low protection and plasmids expressing only parts of VP2 or VP3 are not protective (Mikalsen et al., 2004). This might indicate that posttranslational proteolytic processing of the polyprotein is important for inducing immunogenic protection. Another study shows that in addition to the production of specific antibodies at 30 days post vaccination, also early immune responses are induced by expression of the IPNV VP2 in brown trout. *In vitro* (BF-2 cells) VP2 expression induces an antiviral state protective against homlogous (IPNV) and heterologous (IHNV) viruses. In DNA-vaccines adjuvant factors may be present in the expression vector in the form of CpG motifs which can contribute to immunostimulatory responses. These results suggest that the VP2 gene is a potential candidate for the design of DNA-vaccines, although adjuvant factors and the acquisition of posttranslational modifications must be considered (de las Heras et al., In press).

The antagonistic effect of IPNV

Most viruses have evolved strategies to evade host immune responses in order to promote own existence. IPNV is a sucessful pathogen with a wide specter of susceptible hosts, indicating that the virus has the potential to escape diverse immune mechanisms. The inability

of IPNV to induce IFN synthesis and thereby ISGs such as Mx in certain cell types support our proposed theory that the virus possess a mechanism to circumvent the initial offset of danger-signaling in the host cell. Whether the binding of VP3 to the genomic dsRNA is a mechanism for IPNV to avoid IFN induction has yet to be proven. The IPN virus is also able to down-regulate the induction of ISGs which is activated by exogenously administered IFN (paper 2), suggesting yet another mechanism involved in signaling pathway downstream of IFN induction. We have suggested VP4 and VP5 to be candidate molecules participating in this action, although we have not been able to pinpoint the exact stage this occurs and which host molecules they target. More factors involved in IFN signaling need to be characterized in fish, and their expression and mode of action need to be clarified in the different cell-lines we utilize as tools. Several viruses target STAT or JAK proteins for degradation (Didcock et al., 1999; Parisien et al., 2001; Parisien et al., 2002) or otherwise inhibit their activation (Polyak et al., 2001). Neither of the five virally encoded proteins VP1-VP5 of IPNV were able to interact directly with ssSTAT1a in a Y2H assay (results not shown), predicting that the target for the antagonistic effect of this virus is either an intermediate binding partner to STAT1 or another player in the IFN signaling pathway. Interestingly, we have preliminary data showing that IPNV has the ability to impair IFN-a1 (formerly named IFN-α1) induced phosphorylation of STAT1, whereas IFNγ-induced phosphorylation of STAT1 is not affected (own unpublished results). The effect of each individual viral protein in this inhibition of STAT1 activation/phosphorylation is currently being examined. Screening an Y2H library with the different IPNV proteins as bait might identify other host molecules as putative targets for the antagonistic effect of the virus. Additionally, the interacting ability of activated STAT1 needs to be elucidated.

The role of STAT1

The ssSTAT1a was initially cloned and used in a two-hybrid matrix in a direct approach to identify protein partners interacting with IPNV proteins. No positive interaction was identified, although the characterization of STAT1 as a key molecule in the JAK/STAT pathway was pursued further (paper 3). During this work two other salmon STAT1 genes were published (Collet et al., 2008) or submitted to GenBank (accession no. BT045567). The existence of more than one isotype of STAT1, with distinct transcriptional activation domains could indicate selective transcriptional activation upon stimulation.

Expression levels of STAT

The levels of STAT1 mRNA is previously shown to increase slightly 2 – 4 days after IPNV infection, and as late as 5 – 6 days after ISAV infection. The latter with a more potent induction than IPNV (Collet et al., 2008). *In vitro* studies of ISAV infected TO cells shows increased STAT1 transcription, although at much earlier timepoints (24 – 84 h p.i.) than detected by *in vivo* infection (Kileng et al., 2009). The same authors show that Poly I:C, like IPNV, is a weak inducer of STAT1. Contradicting results are reported by Collet et al. (2007) where STAT1 in RTG-P1 cells seem to be highly inducible by Poly I:C after 7 – 24 h in a semiquantitative RT-PCR. These studies are performed in different salmonid cell-lines, offering an explanation for the discrepancy of the results upon the same stimulation. Data presented in this thesis (paper 3) show only a modest increase in the expression of STAT1 at early time-points in head kidney leukocytes, CHSE-214 and TO cells subjected to IFN-stimulation or IPNV and ISAV infection. However STAT1 was present at a basal level at all times. The primers we used for the qPCR are identical to the primers used by Collet et al. (2007), while they differ from the primers used by Kileng et al. (2009), which are based on the GenBank accession no. BT045567 sequence. These expression patterns are consistent with a constitutively expressed STAT1 which is activated upon certain stimuli, and secondarily up-regulated in a positive feedback loop.

Regulation of STAT1 activation

We show in paper 3 that salmon STAT1 is tyrosine phosphorylated in response to IFN stimulation. In addition there are diverse posttranslational modifications occurring in STAT proteins in mammals; serine phosphorylation (Varinou et al., 2003), acetylation (DNA association and transcriptional activation), methylation (transcriptional activation) (Altschuler et al., 1999; Mowen et al., 2001), ISGylation (ISG15 conjugates to JAK1 and STAT1 and acts as a positive regulator of IFN signaling) (Malakhova et al., 2003) and SUMOylation (small ubiquitin-related modifier covalently linked to STAT1 by PIAS E3 ligase, acts negative on transcriptional activation) (Ungureanu et al., 2003). None of these are yet described in fish. Tyrosine phosphatases are important in order to dephosphorylate STATs and attenuate the signal transduction after the transient STAT activation. SH2-containing phosphatates can act in the cytoplasm by dephosphorylating phosphotyrosines on molecules docked to the IFNAR or JAK, and thereby limit signal transduction in an early phase (David et al., 1995). PIAS

(protein inhibitor of activated STAT1) proteins are constitutively expressed nuclear proteins that interacts with activated STAT1 and suppresses the DNA-binding ability. Thus, these molecules block STATs from acting as transcriptional activators at their target promoter sequences (Liu et al., 1998; Liu et al., 2004). Other inhibitors of cytokine signaling have been described, such as suppressor of cytokine signaling (SOCS) that associate with IFNAR/IFNGR or JAKs and target signaling proteins for ubiquitin/proteasome-mediated degradation (Alexander et al., 1999; Marine et al., 1999).

As many as 11 type I IFN genes, whereof many encode similar IFNs, have recently been identified in salmon (Sun et al., 2009). It is speculated whether the evolution of multiple IFN genes and the need for such massive IFN production is coupled to the salmon being an anadromous species which is exposed to a greater diversity of viruses in multiple environments. What do many different IFNs mean for the diversity and specificity of STAT signaling? The specificity of STAT activation is likely to arise from specific interaction between STATs' SH2 domains and the receptor's phosphotyrosine motifs rather than being determined by the activated JAKs. Therby, a certain level of specificity of cytokine action is allowed. The different kinases involved in this pathway can substitute for each other (Strobl et al., 2001). The different cytokines seem to have preferences as to which STAT they activate. One exception is IL-6 which normally activates STAT3 in mice, but in STAT3 knockout mice is able to activate STAT1 and trigger the typical IFNγ-inducible genes, showing cross-regulation among STATs. In normal cells a STAT3-dependent mechanism would down-regulate levels of STAT1 expression (Costa-Pereira et al., 2002). The specificity of the signal in cases where multiple cytokines activate common STATs must be determined by the cellular context and the specificity in the binding to the promoter-elements and co-activators. STAT1(-/-) mice are extremely susceptible to viral disease and cells and tissues from these mice are unresponsive to IFN, but remain responsive to other cytokines. Thus, STAT1 appears to be specific for IFN pathways (Durbin et al., 1996) and has less aptitude to cross-regulation than STAT3.

Future directions

- Screening of Y2H library from immune-stimulated Atlantic salmon leukocytes with IPNV proteins
- Functional studies of STAT2 and further functional studies of STAT1 in response to IPNV
- Studies of the promoter elements of salmon ISGs – Are they binding STAT?
- Utilize inhibitors of JAK/STAT signaling to pinpoint interference with IPNV
- Explore RNA-binding properties of VP3 – importance in IFN induction/signaling and viral propagation.

Further elucidation of these issues will extend the knowledge of virus-host interaction in fish at molecular levels. Emerging results from high throughput genomic-scale techniques, such as microarrays and proteomics in combination with bioinformatics can be extensively used to discover and analyze components of pathways, targets and strategies of the immune system. Such knowledge can eventually be used in the development of vaccines, medicine and prophylactic strategies to combat virus infections in farmed fish.

Main conclusions

Viral protein interactions

- IPNV VP3 is a key organizer in the viral particle as it has multiple interaction partners: VP3, VP1 and ds RNA.
- VP3 binding to dsRNA is sequence-independent.
- The binding between VP3 and VP1 is independent on the presence of dsRNA.
- VP1-VP3 complexes occur prior to the formation of mature virions indicating a prime role in the assembly process.

Antiviral effect of IFN

- Type I IFN-treatment prior to IPNV infection induces an antiviral state wherein viral protein synthesis halts and VP2 maturation is inhibited.

Viral IFN antagonism

- The IPNV has evolved strategies to evade host immune responses.
- Infection with IPNV prior to IFN-treatment gives higher viral titers, faster viral protein synthesis and reduced expression of antiviral genes.
- VP4 and VP5 are potential candidates involved in down-regulation of IFN-signaling in the JAK/STAT pathway

Salmon STAT1

- A functional isoform of STAT1 from Atlantic salmon (ssSTAT1a) has been cloned.
- ssSTAT1a interacts with itself.
- ssSTAT1a is phosphorylated upon stimulation with type I and type II IFNs.
- Salmon STAT1 translocates to the nucleus within 1 hour upon IFNγ stimulation in certain cell-types.

References

Alexander, W.S., Starr, R., Fenner, J.E., Scott, G.L., Handman, E., Sprigg, N.S., Corbin, J.E., Cornish, A.L., Darwiche, R., Owczarek, C.M., Kay, T.W.H., Nicola, N.A., Hertzog, P.J., Metcalf, D. and Hilton, D.J. (1999) SOCS1 is a critical inhibitor of interferon gamma signaling and prevents the potentially fatal neonatal actions of this cytokine. Cell 98(5), 597-608.

Alexopoulou, L., Holt, A.C., Medzhitov, R. and Flavell, R.A. (2001) Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 413(6857), 732-8.

Allnutt, F.C.T., Bowers, R.M., Rowe, C.G., Vakharia, V.N., LaPatra, S.E. and Dhar, A.K. (2007) Antigenicity of infectious pancreatic necrosis virus VP2 subviral particles expressed in yeast. Vaccine 25(26), 4880-4888.

Altschuler, L., Wook, J.-O., Gurari, D., Chebath, J. and Revel, M. (1999) Involvement of Receptor-Bound Protein Methyltransferase PRMT1 in Antiviral and Antiproliferative Effects of Type I Interferons. Journal of Interferon & Cytokine Research 19(2), 189-195.

Andrejeva, J., Childs, K.S., Young, D.F., Carlos, T.S., Stock, N., Goodbourn, S. and Randall, R.E. (2004) The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the IFN-beta promoter. Proc Natl Acad Sci U S A 101(49), 17264-9.

Babiuk, L.A., van Drunen Littel-van den Hurk, S., Loehr, B.I. and Uwiera, R. (2000) Veterinary applications of DNA vaccines. Dev Biol (Basel) 104, 73-81.

Baeck, G.-W., Kim, J.-W. and Park, C.-I. (2008) Identification and expression analysis of an interferon stimulated gene 15 (ISG15) from black rockfish, Sebastes schlegeli. Fish & Shellfish Immunology 25(5), 679-681.

Bass, B.L. and Weintraub, H. (1988) An unwinding activity that covalently modifies its double-stranded RNA substrate. Cell 55(6), 1089-1098.

Bergan, V., Jagus, R., Lauksund, S., Kileng, O. and Robertsen, B. (2008) The Atlantic salmon Z-DNA binding protein kinase phosphorylates translation initiation factor 2 alpha and constitutes a unique orthologue to the mammalian dsRNA-activated protein kinase R. Febs Journal 275(1), 184-197.

Biacchesi, S., LeBerre, M., Lamoureux, A., Louise, Y., Lauret, E., Boudinot, P. and Bremont, M. (2009) MAVS Plays a Major Role in Induction of the Fish Innate Immune Response against RNA and DNA Viruses. J. Virol., JVI.00404-09.

Birghan, C., Mundt, E. and Gorbalenya, A.E. (2000) A non-canonical lon proteinase lacking the ATPase domain employs the ser-Lys catalytic dyad to exercise broad control over the life cycle of a double-stranded RNA virus. Embo J 19(1), 114-23.

Biron, C.A., and G.C. Sen. (2001) Interferons and Other Cytokines. In: W.W. Lippincott (Ed), Fields Virology and Fundamental Virology, Fourth Edition ed., pp. 321-351, Philadelphia.

Blake, S., Ma, J.Y., Caporale, D.A., Jairath, S. and Nicholson, B.L. (2001) Phylogenetic relationships of aquatic birnaviruses based on deduced amino acid sequences of genome segment A cDNA. Dis Aquat Organ 45(2), 89-102.

Blake, S., Schill, W., McAllister, P., Lee, M., Singer, J. and Nicholson, B. (1995) Detection and identification of aquatic birnaviruses by PCR assay. J. Clin. Microbiol. 33(4), 835-839.

Blindenbacher, A., Duong, F.H.T., Hunziker, L., Stutvoet, S.T.D., Wang, X.Y., Terracciano, L., Moradpour, D., Blum, H.E., Alonzi, T., Tripodi, M., La Monica, N. and Heim,

M.H. (2003) Expression of hepatitis C virus proteins inhibits interferon a signaling in the liver of transgenic mice. Gastroenterology 124(5), 1465-1475.
Borden, E.C., Sen, G.C., Uze, G., Silverman, R.H., Ransohoff, R.M., Foster, G.R. and Stark, G.R. (2007) Interferons at age 50: past, current and future impact on biomedicine. 6(12), 975-990.
Boudinot, P., Massin, P., Blanco, M., Riffault, S. and Benmansour, A. (1999) vig-1, a new fish gene induced by the rhabdovirus glycoprotein, has a virus-induced homologue in humans and shares conserved motifs with the MoaA family. Journal of Virology 73, 1846-52.
Boudinot, P., Salhi, S., Blanco, M. and Benmansour, A. (2001) Viral haemorrhagic septicaemia virus induces vig-2, a new interferon- responsive gene in rainbow trout. Fish Shellfish Immunol 11(5), 383-97.
Bowers, R.M., Lapatra, S.E. and Dhar, A.K. (2008) Detection and quantitation of infectious pancreatic necrosis virus by real-time reverse transcriptase-polymerase chain reaction using lethal and non-lethal tissue sampling. Journal of Virological Methods 147(2), 226-234.
Brierley, M.M. and Fish, E.N. (2002) IFN-alpha/beta receptor interactions to biologic outcomes: Understanding the circuitry. Journal of Interferon and Cytokine Research 22(8), 835-845.
Brierley, M.M. and Fish, E.N. (2005) Stats: Multifaceted regulators of transcription. Journal of Interferon and Cytokine Research 25(12), 733-744.
Caipang, C.M.A., Hirono, I. and Aoki, T. (2003) In vitro inhibition of fish rhabdoviruses by Japanese flounder, Paralichthys olivaceus Mx. Virology 317(2), 373-382.
Caipang, C.M.A., Hirono, I. and Aoki, T. (2005) Induction of antiviral state in fish cells by Japanese flounder, Paralichthys olivaceus, interferon regulatory factor-1. Fish & Shellfish Immunology 19(1), 79-91.
Casañas, A., Navarro, A., Ferrer-Orta, C., González, D., Rodríguez, J.F. and Verdaguer, N. (2008) Structural Insights into the Multifunctional Protein VP3 of Birnaviruses. Structure 16(1), 29-37.
Casani, D., Randelli, E., Costantini, S., Facchiano, A.M., Zou, J., Martin, S., Secombes, C.J., Scapigliati, G. and Buonocore, F. (2009) Molecular characterisation and structural analysis of an interferon homologue in sea bass (Dicentrarchus labrax L.). Molecular Immunology 46(5), 943-952.
Caswell-Reno, P., Reno, P.W. and Nicholson, B.L. (1986) Monoclonal antibodies to infectious pancreatic necrosis virus: analysis of viral epitopes and comparison of different isolates. J Gen Virol 67 (Pt 10), 2193-205.
Chang, H.W., Watson, J.C. and Jacobs, B.L. (1992) The E3l Gene of Vaccinia Virus Encodes an Inhibitor of the Interferon-Induced, Double-Stranded Rna-Dependent Protein-Kinase. Proceedings of the National Academy of Sciences of the United States of America 89(11), 4825-4829.
Christie, K.E. (1997) Immunization with viral antigens: infectious pancreatic necrosis. Dev Biol Stand 90, 191-9.
Cohen, J. (1975) Ribonucleic-Acid Polymerase-Activity in Purified Infectious Pancreatic Necrosis Virus of Trout. Biochemical and Biophysical Research Communications 62(3), 689-695.
Collet, B., Bain, N., Prevost, S., Besinque, G., McBeath, A., Snow, M. and Collins, C. (2008) Isolation of an Atlantic salmon (Salmo salar) signal transducer and activator of transcription STAT1 gene: Kinetics of expression upon ISAV or IPNV infection. Fish & Shellfish Immunology 25(6), 861-867.

Collet, B., Ganne, G., Bird, S. and Collins, C.M. (2009) Isolation and expression profile of a gene encoding for the Signal Transducer and Activator of Transcription STAT2 in Atlantic salmon (Salmo salar). Developmental & Comparative Immunology In Press, Corrected Proof.

Collet, B., Munro, E.S., Gahlawat, S., Acosta, F., Garcia, J., Roemelt, C., Zou, J., Secombes, C.J. and Ellis, A.E. (2007) Infectious pancreatic necrosis virus suppresses type I interferon signalling in rainbow trout gonad cell line but not in Atlantic salmon macrophages. Fish & Shellfish Immunology 22(1-2), 44-56.

Collet, B. and Secombes, C.J. (2001) The rainbow trout (Oncorhynchus mykiss) Mx1 promoter. Structural and functional characterization. European Journal of Biochemistry 268(6), 1577-1584.

Collet, B. and Secombes, C.J. (2002) Type I-interferon signalling in fish. Fish Shellfish Immunol 12(5), 389-97.

Cortés-San Martin, M., Villanueva, R.A., Jashés, M. and Sandino, A.M. (In press) Molecular characterization of IPNV RNA replication intermediates during the viral infective cycle. Virus Research In Press, Accepted Manuscript.

Costa-Pereira, A.P., Tininini, S., Strobl, B., Alonzi, T., Schlaak, J.F., Is'harc, H., Gesualdo, I., Newman, S.J., Kerr, I.M. and Poli, V. (2002) Mutational switch of an IL-6 response to an interferon-gamma-like response. Proceedings of the National Academy of Sciences of the United States of America 99(12), 8043-8047.

Da Costa, B., Chevalier, C., Henry, C., Huet, J.C., Petit, S., Lepault, J., Boot, H. and Delmas, B. (2002) The capsid of infectious bursal disease virus contains several small peptides arising from the maturation process of pVP2. J Virol 76(5), 2393-402.

Darnell, J.E. (1997) Phosphotyrosine signaling and the single cell:metazoan boundary. Proceedings of the National Academy of Sciences of the United States of America 94(22), 11767-11769.

David, M., Chen, H., Goelz, S., Larner, A. and Neel, B. (1995) Differential regulation of the alpha/beta interferon-stimulated Jak/Stat pathway by the SH2 domain-containing tyrosine phosphatase SHPTP1. Mol. Cell. Biol. 15(12), 7050-7058.

de las Heras, A.I., Pérez Prieto, S.I. and Saint-Jean, S.R. (In press) In vitro and in vivo immune responses induced by a DNA vaccine encoding the vp2 gene of the infectious pancreatic necrosis virus. Fish & Shellfish Immunology In Press, Accepted Manuscript.

de las Heras, A.I., Saint-Jean, S.R. and Perez-Prieto, S.I. (2008) Salmonid fish viruses and cell interactions at early steps of the infective cycle. Journal of Fish Diseases 31(7), 535-546.

Didcock, L., Young, D.F., Goodbourn, S. and Randall, R.E. (1999) The V protein of simian virus 5 inhibits interferon signalling by targeting STAT1 for proteasome-mediated degradation. J Virol 73(12), 9928-33.

Dobos, P. (1995) The molecular biology of infectious pancreatic necrosis virus (IPNV). Annual Reviews of Fish Diseases 5, 25-54.

Dorson, M. and Torchy, C. (1985) Experimental transmission of infectious pancreatic necrosis virus via the sexual products. Fish and Shellfish Pathology, 251–260.

Duncan, R. and Dobos, P. (1986) The nucleotide sequence of infectious pancreatic necrosis virus (IPNV) dsRNA segment A reveals one large ORF encoding a precursor polyprotein. Nucleic Acids Res 14(14), 5934.

Duncan, R., Nagy, E., Krell, P.J. and Dobos, P. (1987) Synthesis of the infectious pancreatic necrosis virus polyprotein, detection of a virus-encoded protease, and fine structure mapping of genome segment A coding regions. J Virol 61(12), 3655-64.

Durbin, J.E., Hackenmiller, R., Simon, M.C. and Levy, D.E. (1996) Targeted disruption of the mouse STAT1 results in compromised innate immunity to viral disease. Cell 84(3), 443-450.
Feldman, A.R., Lee, J., Delmas, B. and Paetzel, M. (2006) Crystal Structure of a Novel Viral Protease with a Serine/Lysine Catalytic Dyad Mechanism. Journal of Molecular Biology 358(5), 1378-1389.
Fenner, B.J., Goh, W. and Kwang, J. (2006) Sequestration and Protection of Double-Stranded RNA by the Betanodavirus B2 Protein. J. Virol. 80(14), 6822-6833.
Fernandez-Trujillo, M.A., Garcia-Rosado, E., Alonso, M.C., Borrego, J.J., Alvare, M.C. and Bejar, J. (2008) In vitro inhibition of sole aquabirnavirus by Senegalese sole Mx. Fish & Shellfish Immunology 24(2), 187-193.
Fridholm, H., Eliasson, L. and Everitt, E. (2007) Immunogenicity Properties of Authentic and Heterologously Synthesized Structural Protein VP2 of Infectious Pancreatic Necrosis Virus. Viral Immunology 20(4), 635-648.
Frost, P., Havarstein, L.S., Lygren, B., Stahl, S., Endresen, C. and Christie, K.E. (1995) Mapping of neutralization epitopes on infectious pancreatic necrosis viruses. J Gen Virol 76 (Pt 5), 1165-72.
Frost, P. and Ness, A. (1997) Vaccination of Atlantic salmon with recombinant VP2 of infectious pancreatic necrosis virus (IPNV), added to a multivalent vaccine, suppresses viral replication following IPNV challenge. Fish & Shellfish Immunology 7(8), 609-619.
Gale, M., Jr. and Katze, M.G. (1998) Molecular mechanisms of interferon resistance mediated by viral-directed inhibition of PKR, the interferon-induced protein kinase. Pharmacol Ther 78(1), 29-46.
Gao, Q., Nie, P., Thompson, K.D., Adams, A., Wang, T., Secombes, C.J. and Zou, J. (2009) The search for the IFN-[gamma] receptor in fish: Functional and expression analysis of putative binding and signalling chains in rainbow trout Oncorhynchus mykiss. Developmental & Comparative Immunology In Press, Uncorrected Proof.
Garcia, M.A., Gil, J., Ventoso, I., Guerra, S., Domingo, E., Rivas, C. and Esteban, M. (2006) Impact of protein kinase PKR in cell biology: from antiviral to antiproliferative action. Microbiology and Molecular Biology Reviews 70(4), 1032-+.
García-Rosado, E., Markussen, T., Kileng, Ø., Baekkevold, E.S., Robertsen, B., Mjaaland, S. and Rimstad, E. (2008) Molecular and functional characterization of two infectious salmon anaemia virus (ISAV) proteins with type I interferon antagonizing activity. Virus Research 133(2), 228-238.
Garcia-Sastre, A. and Biron, C.A. (2006) Type 1 interferons and the virus-host relationship: A lesson in detente. Science 312(5775), 879-882.
Garriga, D., Navarro, A., Querol-Audi, J., Abaitua, F., Rodriguez, J.F. and Verdaguer, N. (2007) Activation mechanism of a noncanonical RNA-dependent RNA polymerase. Proceedings of the National Academy of Sciences 104(51), 20540-20545.
George, C.X. and Samuel, C.E. (1999) Human RNA-specific adenosine deaminase ADAR1 transcripts possess alternative exon 1 structures that initiate from different promoters, one constitutively active and the other interferon inducible. Proceedings of the National Academy of Sciences of the United States of America 96(8), 4621-4626.
Granzow, H., Weiland, F., Fichtner, D. and Enzmann, P.J. (1997) Studies of the ultrastructure and morphogenesis of fish pathogenic viruses grown in cell culture. Journal of Fish Diseases 20(1), 1-10.
Grayfer, L. and Belosevic, M. (2009) Molecular characterization, expression and functional analysis of goldfish (Carassius aurutus L.) interferon gamma. Developmental & Comparative Immunology 33(2), 235-246.

Guy, D.R., Bishop, S.C., Brotherstone, S., Hamilton, A., Roberts, R.J., McAndrew, B.J. and Woolliams, J.A. (2006) Analysis of the incidence of infectious pancreatic necrosis mortality in pedigreed Atlantic salmon, Salmo salar L., populations. Journal of Fish Diseases 29(11), 637-647.

Haller, O., Frese, M. and Kochs, G. (1998) Mx proteins: mediators of innate resistance to RNA viruses. Revue Scientifique Et Technique De L Office International Des Epizooties 17(1), 220-230.

Haller, O., Kochs, G. and Weber, F. (2006) The interferon response circuit: induction and suppression by pathogenic viruses. Virology 344(1), 119-30.

Heppell, J., Tarrab, E., Lecomte, J., Berthiaume, L. and Arella, M. (1995) Strain Variability and Localization of Important Epitopes on the Major Structural Protein (VP2) of Infectious Pancreatic Necrosis Virus1. Virology 214(1), 40-49.

Hiscott, J., Lacoste, J. and Lin, R.T. (2006a) Recruitment of an interferon molecular signaling complex to the mitochondrial membrane: Disruption by hepatitis C virus NS3-4A protease. Biochemical Pharmacology 72(11), 1477-1484.

Hiscott, J., Lin, R., Nakhaei, P. and Paz, S. (2006b) MasterCARD: a priceless link to innate immunity. Trends in Molecular Medicine 12(2), 53-56.

Holland, J.W., Bird, S., Williamson, B., Woudstra, C., Mustafa, A., Wang, T., Zou, J., Blaney, S.C., Collet, B. and Secombes, C.J. (2008) Molecular characterization of IRF3 and IRF7 in rainbow trout, Oncorhynchus mykiss: Functional analysis and transcriptional modulation. Molecular Immunology 46(2), 269-285.

Hong, J.R., Gong, H.Y. and Wu, J.L. (2002) IPNV VP5, a novel anti-apoptosis gene of the Bcl-2 family, regulates Mcl-1 and viral protein expression. Virology 295(2), 217-29.

Hong, J.-R., Guan, B.-J., Her, G.M., Evensen, O., Santi, N. and Wu, J.-L. (2008) Aquatic birnavirus infection activates the transcription factor NF-kappaB via tyrosine kinase signalling leading to cell death. Journal of Fish Diseases 31(6), 451-460.

Houston, R.D., Gheyas, A., Hamilton, A., Guy, D.R., Tinch, A.E., Taggart, J.B., McAndrew, B.J., Haley, C.S. and Bishop, S.C. (2008) Detection and Confirmation of a Major QTL Affecting Resistance to Infectious Pancreatic Necrosis (IPN) in Atlantic Salmon (Salmo Salar). Animal Genomics for Animal Health 132, 199-204.

Hovanessian, A.G. (2007) On the discovery of interferon-inducible, double-stranded RNA activated enzymes: The 2'-5'-oligoadenylate synthetases and the protein kinase PKR. Cytokine & growth factor reviews 18(5), 351-361.

Hu, C.Y., Zhang, Y.B., Huang, G.P., Zhang, Q.Y. and Gui, J.F. (2004) Molecular cloning and characterisation of a fish PKR-like gene from cultured CAB cells induced by UV-inactivated virus. Fish Shellfish Immunol 17(4), 353-66.

Hwang, H.J., Moon, C.H., Kim, H.G., Kim, J.Y., Lee, J.M., Park, J.W. and Chung, D.K. (2007) Identification and functional analysis of salmon annexin 1 induced by a virus infection in a fish cell line. Journal of Virology 81(24), 13816-13824.

Igawa, D., Sakai, M. and Savan, R. (2006) An unexpected discovery of two interferon gamma-like genes along with interleukin (IL)-22 and -26 from teleost: IL-22 and -26 genes have been described for the first time outside mammals. Molecular Immunology 43(7), 999-1009.

Imajoh, M., Yagyu, K. and Oshima, S. (2003) Early interactions of marine birnavirus infection in several fish cell lines. Journal of General Virology 84, 1809-1816.

Ingerslev, H.-C., Rønneseth, A., Pettersen, E.F. and Wergeland, H.I. (2009) Differential Expression of Immune Genes in Atlantic Salmon (*Salmo salar* L.) Challenged Intraperitoneally or by Cohabitation with IPNV. Scandinavian Journal of Immunology 69(2), 90-98.

Inoue, Y., Kamota, S., Ito, K., Yoshiura, Y., Ototake, M., Moritomo, T. and Nakanishi, T. (2005) Molecular cloning and expression analysis of rainbow trout (Oncorhynchus mykiss) interleukin-10 cDNAs. Fish & Shellfish Immunology 18(4), 335-344.
Isaacs, A. and Lindenmann, J. (1957) Virus Interference .1. The Interferon. Proceedings of the Royal Society of London Series B-Biological Sciences 147(927), 258-267.
Jault, C., Pichon, L. and Chluba, J. (2004) Toll-like receptor gene family and TIR-domain adapters in Danio rerio. Molecular Immunology 40(11), 759-771.
Jensen, I. and Robertsen, B. (2002) Effect of double-stranded RNA and interferon on the antiviral activity of Atlantic salmon cells against infectious salmon anemia virus and infectious pancreatic necrosis virus. Fish Shellfish Immunol 13(3), 221-41.
Jin, H.K., Yoshimatsu, K., Takada, A., Ogino, M., Asano, A., Arikawa, J. and Watanabe, T. (2001) Mouse Mx2 protein inhibits hantavirus but not influenza virus replication. Archives of Virology 146(1), 41-49.
Johansen, A., Collet, B., Sandaker, E., Secombes, C.J. and Jorgensen, J.B. (2004) Quantification of Atlantic salmon type-I interferon using an Mx1 promoter reporter gene assay. Fish Shellfish Immunol 16(2), 173-84.
Jørgensen, J.B., Johansen, A., Hegseth, M.N., Zou, J., Robertsen, B., Collet, B. and Secombes, C.J. (2007) A recombinant CHSE-214 cell line expressing an Mx1 promoter-reporter system responds to both interferon type I and type II from salmonids and represents a versatile tool to study the IFN-system in teleost fish. Fish & Shellfish Immunology 23(6), 1294-1303.
Kato, H., Sato, S., Yoneyama, M., Yamamoto, M., Uematsu, S., Matsui, K., Tsujimura, T., Takeda, K., Fujita, T., Takeuchi, O. and Akira, S. (2005) Cell Type-Specific Involvement of RIG-I in Antiviral Response. Immunity 23(1), 19-28.
Katze, M.G., He, Y. and Gale, M., Jr. (2002) Viruses and interferon: a fight for supremacy. Nat Rev Immunol 2(9), 675-87.
Kawai, T. and Akira, S. (2005) Pathogen recognition with Toll-like receptors. Current Opinion in Immunology 17(4), 338-344.
Kawai, T. and Akira, S. (2006) TLR signaling. 13(5), 816-825.
Kawai, T. and Akira, S. (2008) Toll-like Receptor and RIG-1-like Receptor Signaling, Year in Immunology 2008, pp. 1-20. Vol. 1143. BLACKWELL PUBLISHING, Oxford.
Kawai, T. and Akira, S. (2009) The roles of TLRs, RLRs and NLRs in pathogen recognition. Int. Immunol. 21(4), 317-337.
Kileng, Ø., Bergan, V., Workenhe, S.T. and Robertsen, B. (2009) Structural and functional studies of an IRF-7-like gene from Atlantic salmon. Developmental & Comparative Immunology 33(1), 18-27.
Kim, H.J., Oseko, N., Nishizawa, T. and Yoshimizu, M. (2009) Protection of rainbow trout from infectious hematopoietic necrosis (IHN) by injection of infectious pancreatic necrosis virus (IPNV) or Poly(I:C). Diseases of Aquatic Organisms 83(2), 105-113.
Kuznar, J., Soler, M., Farias, G. and Espinoza, J.C. (1995) Attachment and Entry of Infectious Pancreatic Necrosis Virus (Ipnv) into Chse-214 Cells. Archives of Virology 140(10), 1833-1840.
Lai, F., Drakas, R. and Nishikura, K. (1995) Mutagenic Analysis of Double-Stranded-Rna Adenosine-Deaminase, a Candidate Enzyme for Rna Editing of Glutamate-Gated Ion-Channel Transcripts. Journal of Biological Chemistry 270(29), 17098-17105.
Langland, J.O., Pettiford, S., Jiang, B.M. and Jacobs, B.L. (1994) Products of the Porcine Group-C Rotavirus Nsp3 Gene Bind Specifically to Double-Stranded-Rna and Inhibit Activation of the Interferon-Induced Protein-Kinase Pkr. Journal of Virology 68(6), 3821-3829.

Lannan, C.N., Winton, J.R. and Fryer, J.L. (1984) Fish cell lines: Establishment and characterization of nine cell lines from salmonids. IN VITRO. Vol. 20, no. 9.
Larsen, R., Rokenes, T.P. and Robertsen, B. (2004) Inhibition of infectious pancreatic necrosis virus replication by atlantic salmon Mx1 protein. J Virol 78(15), 7938-44.
Lee, J., Feldman, A.R., Delmas, B. and Paetzel, M. (2007) Crystal Structure of the VP4 Protease from Infectious Pancreatic Necrosis Virus Reveals the Acyl-Enzyme Complex for an Intermolecular Self-cleavage Reaction. J. Biol. Chem. 282(34), 24928-24937.
Leonard, G.T. and Sen, G.C. (1996) Effects of adenovirus E1A protein on interferon-signaling. Virology 224(1), 25-33.
Leu, J.-H., Chang, M.-S., Yao, C.-W., Chou, C.-K., Chen, S.-T. and Huang, C.-J. (1998) Genomic organization and characterization of the promoter region of the round-spotted pufferfish (Tetraodon fluviatilis) JAK1 kinase gene. Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression 1395(1), 50-56.
Leu, J.H., Yan, S.J., Lee, T.F., Chou, C.M., Chen, S.T., Hwang, P.P., Chou, C.K. and Huang, C.J. (2000) Complete genomic organization and promoter analysis of the round-spotted pufferfish JAK1, JAK2, JAK3, and TYK2 genes. DNA and Cell Biology 19(7), 431-446.
Levraud, J.-P., Boudinot, P., Colin, I., Benmansour, A., Peyrieras, N., Herbomel, P. and Lutfalla, G. (2007) Identification of the Zebrafish IFN Receptor: Implications for the Origin of the Vertebrate IFN System. J Immunol 178(7), 4385-4394.
Levy, D.E. and Darnell, J.E. (2002) STATs: transcriptional control and biological impact. 3(9), 651-662.
Lin, C.H., John, J.A.C. and Chang, C.Y. (2006) Inhibition of nervous necrosis virus propagation by fish Mx proteins. Biochemical and Biophysical Research Communications 351(2), 534-539.
Liu, B., Liao, J., Rao, X., Kushner, S.A., Chung, C.D., Chang, D.D. and Shuai, K. (1998) Inhibition of Stat1-mediated gene activation by PIAS1
Proceedings of the National Academy of Sciences of the United States of America 95(18), 10626-10631.
Liu, B., Mink, S., Wong, K.A., Stein, N., Getman, C., Dempsey, P.W., Wu, H. and Shuai, K. (2004) PIAS1 selectively inhibits interferon-inducible genes and is important in innate immunity. Nature Immunology 5(9), 891-898.
Liu, Y., George, C.X., Patterson, J.B. and Samuel, C.E. (1997) Functionally distinct double-stranded RNA-binding domains associated with alternative splice site variants of the interferon-inducible double-stranded RNA-specific adenosine deaminase. Journal of Biological Chemistry 272(7), 4419-4428.
Look, D.C., Roswit, W.T., Frick, A.G., Gris-Alevy, Y., Dickhaus, D.M., Walter, M.J. and Holtzman, M.J. (1998) Direct suppression of Stat1 function during adenoviral infection. Immunity 9(6), 871-880.
Lopez-Lastra, M., Gonzalez, M., Jashes, M. and Sandino, A.M. (1994) A detection method for infectious pancreatic necrosis virus (IPNV) based on reverse transcription (RT)-polymerase chain reaction (PCR). Journal of Fish Diseases 17(3), 269-282.
Lopez-Munoz, A., Roca, F.J., Meseguer, J. and Mulero, V. (2009) New Insights into the Evolution of IFNs: Zebrafish Group II IFNs Induce a Rapid and Transient Expression of IFN-Dependent Genes and Display Powerful Antiviral Activities. J Immunol 182(6), 3440-3449.
Lorenzen, N. and LaPatra, S.E. (2005) DNA vaccines for aquacultured fish. Revue Scientifique Et Technique-Office International Des Epizooties 24(1), 201-213.

Lutfalla, G., Crollius, H.R., Stange-thomann, N., Jaillon, O., Mogensen, K. and Monneron, D. (2003) Comparative genomic analysis reveals independent expansion of a lineage-specific gene family in vertebrates: The class II cytokine receptors and their ligands in mammals and fish. Bmc Genomics 4.
Macdonald, A. and Harris, M. (2004) Hepatitis C virus NS5A: tales of a promiscuous protein. Journal of General Virology 85, 2485-2502.
Magyar, G. and Dobos, P. (1994a) Evidence for the detection of the infectious pancreatic necrosis virus polyprotein and the 17-kDa polypeptide in infected cells and of the NS protease in purified virus. Virology 204(2), 580-9.
Magyar, G. and Dobos, P. (1994b) Expression of infectious pancreatic necrosis virus polyprotein and VP1 in insect cells and the detection of the polyprotein in purified virus. Virology 198(2), 437-45.
Maher, S.G., Romero-Weaver, A.L., Scarzello, A.J. and Gamero, A.M. (2007) Interferon: Cellular executioner or white knight? Current Medicinal Chemistry 14(12), 1279-1289.
Malakhova, O.A., Yan, M., Malakhov, M.P., Yuan, Y., Ritchie, K.J., Kim, K.I., Peterson, L.F., Shuai, K. and Zhang, D.-E. (2003) Protein ISGylation modulates the JAK-STAT signaling pathway. Genes & Development 17(4), 455-460.
Maraver, A., Ona, A., Abaitua, F., Gonzalez, D., Clemente, R., Ruiz-Diaz, J.A., Caston, J.R., Pazos, F. and Rodriguez, J.F. (2003) The oligomerization domain of VP3, the scaffolding protein of infectious bursal disease virus, plays a critical role in capsid assembly. J Virol 77(11), 6438-49.
Marine, J.-C., Topham, D.J., McKay, C., Wang, D., Parganas, E., Stravopodis, D., Yoshimura, A. and Ihle, J.N. (1999) SOCS1 Deficiency Causes a Lymphocyte-Dependent Perinatal Lethality. Cell 98(5), 609-616.
Martin, S.A.M., Mohanty, B.P., Cash, P., Houlihan, D.F. and Secombes, C.J. (2007a) Proteome analysis of the Atlantic salmon (Salmo salar) cell line SHK-1 following recombinant IFN- stimulation. PROTEOMICS 7(13), 2275-2286.
Martin, S.A.M., Taggart, J.B., Seear, P., Bron, J.E., Talbot, R., Teale, A.J., Sweeney, G.E., Hoyheim, B., Houlihan, D.F., Tocher, D.R., Zou, J. and Secombes, C.J. (2007b) Interferon type I and type II responses in an Atlantic salmon (Salmo salar) SHK-1 cell line by the salmon TRAITS/SGP microarray. Physiol. Genomics 32(1), 33-44.
Matsuo, A., Oshiumi, H., Tsujita, T., Mitani, H., Kasai, H., Yoshimizu, M., Matsumoto, M. and Seya, T. (2008) Teleost TLR22 Recognizes RNA Duplex to Induce IFN and Protect Cells from Birnaviruses. J Immunol 181(5), 3474-3485.
McAllister, P.E., Owens, W.J. and Ruppenthal, T.M. (1987) Detection of infectious pancreatic necrosis virus in pelleted cell and particulate components from ovarian fluid of brook trout Salvelinus fontinalis. Diseases of Aquatic Organisms. Vol. 2, no. 3.
McBeath, A.J., Collet, B., Paley, R., Duraffour, S., Aspehaug, V., Biering, E., Secombes, C.J. and Snow, M. (2006) Identification of an interferon antagonist protein encoded by segment 7 of infectious salmon anaemia virus. Virus Res 115(2), 176-84.
McBeath, A.J.A., Snow, M., Secombes, C.J., Ellis, A.E. and Collet, B. (2007) Expression kinetics of interferon and interferon-induced genes in Atlantic salmon (Salmo salar) following infection with infectious pancreatic necrosis virus and infectious salmon anaemia virus. Fish & Shellfish Immunology 22(3), 230-241.
McWhirter, S.M., tenOever, B.R. and Maniatis, T. (2005) Connecting Mitochondria and Innate Immunity. Cell 122(5), 645-647.
M'Gonigle, R.H. (1941) Acute cattarhal enteritis of salmonid fingerlings. Transaction of the American Fisheries Society 70, 297-303.

Mikalsen, A.B., Torgersen, J., Alestrom, P., Hellemann, A.L., Koppang, E.O. and Rimstad, E. (2004) Protection of Atlantic salmon Salmo salar against infectious pancreatic necrosis after DNA vaccination. Diseases of Aquatic Organisms 60(1), 11-20.
Milev-Milovanovic, I., Long, S., Wilson, M., Bengten, E., Miller, N.W. and Chinchar, V.G. (2006) Identification and expression analysis of interferon gamma genes in channel catfish. Immunogenetics 58(1), 70-80.
Milne, S.A., Gallacher, S., Cash, P. and Porter, A.J.R. (2006) A reliable RT-PCR-ELISA method for the detection of infectious pancreatic necrosis virus (IPNV) in farmed rainbow trout. Journal of Virological Methods 132(1-2), 92-96.
Mowen, K.A., Tang, J., Zhu, W., Schurter, B.T., Shuai, K., Herschman, H.R. and David, M. (2001) Arginine Methylation of STAT1 Modulates IFN[alpha]/[beta]-Induced Transcription. Cell 104(5), 731-741.
Muller, H., Islam, M.R. and Raue, R. (2003) Research on infectious bursal disease--the past, the present and the future. Vet Microbiol 97(1-2), 153-65.
Munro, E.S. and Ellis, A.E. (2008) A comparison between non-destructive and destructive testing of Atlantic salmon, Salmo salar L., broodfish for IPNV - destructive testing is still the best at time of maturation. Journal of Fish Diseases 31(3), 187-195.
Munro, E.S., Gahlawat, S.K., Acosta, F. and Ellis, A.E. (2006) In infectious pancreatic necrosis virus carrier Atlantic salmon, Salmo salar L., post-smolts, almost all kidney macrophages ex vivo contain a low level of non-replicating virus. Journal of Fish Diseases 29(1), 43-48.
Murray, A.G. (2006) Persistence of infectious pancreatic necrosis virus (IPNV) in Scottish salmon (Salmo salar L.) farms. Preventive Veterinary Medicine 76(1-2), 97-108.
NCBI. (2009) National Center for Biotechnology Information (NCBI). http://www.ncbi.nlm.nih.gov/.
Nie, Y., Hammond, G.L. and Yang, J.-H. (2007) Double-Stranded RNA Deaminase ADAR1 Increases Host Susceptibility to Virus Infection. J. Virol. 81(2), 917-923.
Noga, E.J. (2000) Fish disease: diagnosis and treatment, 367 pp. 2 ed. Wiley-Blackwell.
Oates, A.C., Wollberg, P., Pratt, S.J., Paw, B.H., Johnson, S.L., Ho, R.K., Postlethwait, J.H., Zon, L.I. and Wilks, A.F. (1999) Zebrafish stat3 is expressed in restricted tissues during embryogenesis and stat1 rescues cytokine signaling in a STAT1-deficient human cell line. Developmental Dynamics 215(4), 352-370.
O'Farrell, C., Vaghefi, N., Cantonnet, M., Buteau, B., Boudinot, P. and Benmansour, A. (2002) Survey of transcript expression in rainbow trout leukocytes reveals a major contribution of interferon-responsive genes in the early response to a rhabdovirus infection. J Virol 76(16), 8040-9.
OIE. (2000) Diagnostic Manual for Aquatiq Diseases, 3rd ed. World Organisation for Animal Health, Paris.
Onoguchi, K., Yoneyama, M., Takemura, A., Akira, S., Taniguchi, T., Namiki, H. and Fujita, T. (2007) Viral Infections Activate Types I and III Interferon Genes through a Common Mechanism. J. Biol. Chem. 282(10), 7576-7581.
Ooi, E.L., Hirono, I. and Aoki, T. (2006) Functional characterisation of the Japanese flounder, Paralichthys olivaceus, Mx promoter. Fish & Shellfish Immunology 21(3), 293-304.
Oshiumi, H., Tsujita, T., Shida, K., Matsumoto, M., Ikeo, K. and Seya, T. (2003) Prediction of the prototype of the human Toll-like receptor gene family from the pufferfish, Fugu rubripes, genome. Immunogenetics 54(11), 791-800.
Parisien, J.P., Lau, J.F., Rodriguez, J.J., Sullivan, B.M., Moscona, A., Parks, G.D., Lamb, R.A. and Horvath, C.M. (2001) The V protein of human parainfluenza virus 2 antagonizes type 1 interferon responses by destabilizing signal transducer and activator of transcription 2. Virology 283(2), 230-239.

Parisien, J.P., Lau, J.F., Rodriguez, J.J., Ulane, C.M. and Horvath, C.A. (2002) Selective STAT protein degradation induced by paramyxoviruses requires both STAT1 and STAT2 but is independent of alpha/beta interferon signal transduction. Journal of Virology 76(9), 4190-4198.

Patterson, J.B. and Samuel, C.E. (1995) Expression and Regulation by Interferon of a Double-Stranded-Rna-Specific Adenosine-Deaminase from Human-Cells - Evidence for 2 Forms of the Deaminase. Molecular and Cellular Biology 15(10), 5376-5388.

Patterson, J.B., Thomis, D.C., Hans, S.L. and Samuel, C.E. (1995) Mechanism of Interferon Action: Double-Stranded RNA-Specific Adenosine Deaminase from Human Cells Is Inducible by Alpha and Gamma Interferons. Virology 210(2), 508-511.

Pavlovic, J., Zurcher, T., Haller, O. and Staeheli, P. (1990) Resistance to influenza virus and vesicular stomatitis virus conferred by expression of human MxA protein. J. Virol. 64(7), 3370-3375.

Petit, S., Lejal, N., Huet, J.C. and Delmas, B. (2000) Active residues and viral substrate cleavage sites of the protease of the birnavirus infectious pancreatic necrosis virus. Journal of Virology 74(5), 2057-2066.

Platanias, L.C. (2005) Mechanisms of type-I- and type-II-interferon-mediated signalling. Nat Rev Immunol 5(5), 375-86.

Polyak, S.J., Khabar, K.S.A., Paschal, D.M., Ezelle, H.J., Duverlie, G., Barber, G.N., Levy, D.E., Mukaida, N. and Gretch, D.R. (2001) Hepatitis C virus nonstructural 5A protein induces interleukin-8, leading to partial inhibition of the interferon-induced antiviral response. Journal of Virology 75(13), 6095-6106.

Purcell, M.K., Smith, K.D., Aderem, A., Hood, L., Winton, J.R. and Roach, J.C. (2006) Conservation of Toll-like receptor signaling pathways in teleost fish. Comparative Biochemistry and Physiology D-Genomics & Proteomics 1(1), 77-88.

Ramstad, A. and Midtlyng, P.J. (2008) Strong genetic influence on IPN vaccination-and-challenge trials in Atlantic salmon, *Salmo salar* L. Journal of Fish Diseases 31(8), 567-578.

Randall, R.E. and Goodbourn, S. (2008) Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. Journal of General Virology 89, 1-47.

Roach, J.C., Glusman, G., Rowen, L., Kaur, A., Purcell, M.K., Smith, K.D., Hood, L.E. and Aderem, A. (2005) The evolution of vertebrate Toll-like receptors. Proceedings of the National Academy of Sciences of the United States of America 102(27), 9577-9582.

Roberts, R.J. and Pearson, M.D. (2005) Infectious pancreatic necrosis in Atlantic salmon, *Salmo salar* L. Journal of Fish Diseases 28(7), 383-390.

Robertsen, B. (2006) The interferon system of teleost fish. Fish Shellfish Immunol 20(2), 172-91.

Robertsen, B. (2008) Expression of interferon and interferon-induced genes in salmonids in response to virus infection, interferon-inducing compounds and vaccination. Fish & Shellfish Immunology 25(4), 351-357.

Robertsen, B., Bergan, V., Rokenes, T., Larsen, R. and Albuquerque, A. (2003) Atlantic salmon interferon genes: cloning, sequence analysis, expression, and biological activity. J Interferon Cytokine Res 23(10), 601-12.

Rodriguez, M., Wiens, G., Purcell, M. and Palti, Y. (2005) Characterization of Toll-like receptor 3 gene in rainbow trout (Oncorhynchus mykiss). Immunogenetics 57(7), 510-519.

Rodriguez Saint-Jean, S., Borrego, J.J. and Perez-Prieto, S.I. (2003) Infectious pancreatic necrosis virus: biology, pathogenesis, and diagnostic methods, Adv Virus Res, pp. 113-65. Vol. 62.

Rogatsky, I. and Ivashkiv, L.B. (2006) Glucocorticoid modulation of cytokine signaling. Tissue Antigens 68(1), 1-12.
Rothenburg, S., Deigendesch, N., Dittmar, K., Koch-Nolte, F., Haag, F., Lowenhaupt, K. and Rich, A. (2005) A PKR-like eukaryotic initiation factor 2a kinase from zebrafish contains Z-DNA binding domains instead of dsRNA binding domains. Proceedings of the National Academy of Sciences of the United States of America 102(5), 1602-1607.
Røkenes, T.P., Larsen, R. and Robertsen, B. (2007) Atlantic salmon ISG15: Expression and conjugation to cellular proteins in response to interferon, double-stranded RNA and virus infections. Molecular Immunology 44(5), 950-959.
Sadler, A.J. and Williams, B.R.G. (2008) Interferon-inducible antiviral effectors. 8(7), 559-568.
Saint-Jean, S.R. and Pérez-Prieto, S.I. (2007) Effects of salmonid fish viruses on Mx gene expression and resistance to single or dual viral infections. Fish & Shellfish Immunology 23(2), 390-400.
Salonius, K., Simard, N., Harland, R. and Ulmer, J.B. (2007) The road to licensure of a DNA vaccine. Current Opinion in Investigational Drugs 8(8), 635-641.
Samuel, C.E. (2001) Antiviral actions of interferons. Clin Microbiol Rev 14(4), 778-809.
Santi, N., Sandtro, A., Sindre, H., Song, H., Hong, J.R., Thu, B., Wu, J.L., Vakharia, V.N. and Evensen, O. (2005a) Infectious pancreatic necrosis virus induces apoptosis in vitro and in vivo independent of VP5 expression. Virology 342(1), 13-25.
Santi, N., Song, H., Vakharia, V.N. and Evensen, O. (2005b) Infectious pancreatic necrosis virus VP5 is dispensable for virulence and persistence. J Virol 79(14), 9206-16.
Santi, N., Vakharia, V.N. and Evensen, O. (2004) Identification of putative motifs involved in the virulence of infectious pancreatic necrosis virus. Virology 322(1), 31-40.
Savan, R., Ravichandran, S., Collins, J.R., Sakai, M. and Young, H.A. (2009) Structural conservation of interferon gamma among vertebrates. Cytokine & Growth Factor Reviews 20(2), 115-124.
Scadden, A.D.J. and Smith, C.W.J. (2001) Specific cleavage of hyper-edited dsRNAs. Embo Journal 20(15), 4243-4252.
Schnorr, J.J., Schneider-Schaulies, S., Simon-Jodicke, A., Pavlovic, J., Horisberger, M.A. and ter Meulen, V. (1993) MxA-dependent inhibition of measles virus glycoprotein synthesis in a stably transfected human monocytic cell line. J. Virol. 67(8), 4760-4768.
Seppola, M., Stenvik, J., Steiro, K., Solstad, T., Robertsen, B. and Jensen, I. (2007) Sequence and expression analysis of an interferon stimulated gene (ISG15) from Atlantic cod (Gadus morhua L.). Developmental & Comparative Immunology 31(2), 156-171.
Seth, R.B., Sun, L., Ea, C.-K. and Chen, Z.J. (2005) Identification and Characterization of MAVS, a Mitochondrial Antiviral Signaling Protein that Activates NF-[kappa]B and IRF3. Cell 122(5), 669-682.
Skjæveland, I., Iliev, D.B., Strandskog, G. and Jørgensen, J.B. (2009) Identification and characterization of TLR8 and MyD88 homologs in Atlantic salmon (Salmo salar). Developmental & Comparative Immunology 33(9), 1011-1017.
Skjæveland, I., Iliev, D.B., Zou, J., Jørgensen, T. and Jørgensen, J.B. (2008) A TLR9 homolog that is up-regulated by IFN-[gamma] in Atlantic salmon (Salmo salar). Developmental & Comparative Immunology 32(6), 603-607.
Slavov, D., Crnogorac-Jurcevic, T., Clark, M. and Gardiner, K. (2000) Comparative analysis of the DRADA A-to-I RNA editing gene from mammals, pufferfish and zebrafish. Gene 250(1-2), 53-60.
Smail, D.A. and Munro, E.S. (2008) Isolation and quantification of infectious pancreatic necrosis virus from ovarian and seminal fluids of Atlantic salmon, *Salmo salar* L. Journal of Fish Diseases 31(1), 49-58.

Soliman, H., Midtlyng, P.J. and El-Matbouli, M. (2009) Sensitive and rapid detection of infectious pancreatic necrosis virus by reverse transcription loop mediated isothermal amplification. Journal of Virological Methods 158(1-2), 77-83.
Song, H., Santi, N., Evensen, O. and Vakharia, V.N. (2005) Molecular determinants of infectious pancreatic necrosis virus virulence and cell culture adaptation. J Virol 79(16), 10289-99.
Staeheli, P., Haller, O., Boll, W., Lindenmann, J. and Weissmann, C. (1986) Mx Protein - Constitutive Expression in 3t3-Cells Transformed with Cloned Mx Cdna Confers Selective Resistance to Influenza-Virus. Cell 44(1), 147-158.
Stein, C., Caccamo, M., Laird, G. and Leptin, M. (2007) Conservation and divergence of gene families encoding components of innate immune response systems in zebrafish. Genome Biology 8(11).
Strobl, B., Arulampalam, V., Is'harc, H., Newman, S.J., Schlaak, J.F., Watling, D., Costa-Pereira, A.P., Schaper, F., Behrmann, I., Sheehan, K.C.F., Schreiber, R.D., Horn, F., Heinrich, P.C. and Kerr, I.M. (2001) A completely foreign receptor can mediate an interferon-gamma-like response. Embo Journal 20(19), 5431-5442.
Sun, B., Robertsen, B., Wang, Z. and Liu, B. (2009) Identification of an Atlantic salmon IFN multigene cluster encoding three IFN subtypes with very different expression properties. Developmental & Comparative Immunology 33(4), 547-558.
Sun, Q., Sun, L., Liu, H.-H., Chen, X., Seth, R.B., Forman, J. and Chen, Z.J. (2006) The Specific and Essential Role of MAVS in Antiviral Innate Immune Responses. Immunity 24(5), 633-642.
Tacken, M.G., Peeters, B.P., Thomas, A.A., Rottier, P.J. and Boot, H.J. (2002) Infectious bursal disease virus capsid protein VP3 interacts both with VP1, the RNA-dependent RNA polymerase, and with viral double-stranded RNA. J Virol 76(22), 11301-11.
Taksdal, T., Dannevig, B.H. and Rimstad, E. (2001) Detection of infectious pancreatic necrosis (IPN)-virus in experimentally infected Atlantic salmon parr by RT-PCR and cell culture isolation. Bulletin of the European Association of Fish Pathologists 21(5), 214-219.
Tan, S.L., Nakao, H., He, Y.P., Vijaysri, S., Neddermann, P., Jacobs, B.L., Mayer, B.J. and Katze, M.G. (1999) NS5A, a nonstructural protein of hepatitis C virus, binds growth factor receptor-bound protein 2 adaptor protein in a Src homology 3 domain/ligand-dependent manner and perturbs mitogenic signaling. Proceedings of the National Academy of Sciences of the United States of America 96(10), 5533-5538.
Tarrab, E., Berthiaume, L., Heppell, J., Arella, M. and Lecomte, J. (1993) Antigenic characterization of serogroup 'A' of infectious pancreatic necrosis virus with three panels of monoclonal antibodies. J Gen Virol 74(9), 2025-2030.
Toranzo, A.E. and Hetrick, F.M. (1982) Comparative stability of two salmonid viruses and poliovirus in fresh, estuarine and marine waters. Journal of Fish Diseases 5(3), 223-231.
Ungureanu, D., Vanhatupa, S., Kotaja, N., Yang, J., Aittomaki, S., Janne, O.A., Palvimo, J.J. and Silvennoinen, O. (2003) PIAS proteins promote SUMO-1 conjugation to STAT1. Blood 102(9), 3311-3313.
Unterholzner, L. and Bowie, A.G. (2008) The interplay between viruses and innate immune signaling: Recent insights and therapeutic opportunities. Biochemical Pharmacology 75(3), 589-602.
Varinou, L., Ramsauer, K., Karaghiosoff, M., Kolbe, T., Pfeffer, K., Müller, M. and Decker, T. (2003) Phosphorylation of the Stat1 Transactivation Domain Is Required for Full-Fledged IFN-[gamma]-Dependent Innate Immunity. Immunity 19(6), 793-802.

Villanueva, R.A., Galaz, J.L., Valdes, J.A., Jashes, M.M. and Sandino, A.M. (2004) Genome assembly and particle maturation of the birnavirus infectious pancreatic necrosis virus. J Virol 78(24), 13829-38.

Wallace, I.S., Gregory, A., Murray, A.G., Munro, E.S. and Raynard, R.S. (2008) Distribution of infectious pancreatic necrosis virus (IPNV) in wild marine fish from Scottish waters with respect to clinically infected aquaculture sites producing Atlantic salmon, Salmo salar L. Journal of Fish Diseases 31(3), 177-186.

Wang, F., Barrett, J.W., Shao, Q., Gao, X., Dekaban, G.A. and McFadden, G. (2009) Myxoma virus selectively disrupts type I interferon signaling in primary human fibroblasts by blocking the activation of the Janus kinase Tyk2. Virology 387(1), 136-146.

Wang, X.Y., Li, M., Zheng, H.Y., Muster, T., Palese, P., Beg, A.A. and Garcia-Sastre, A. (2000) Influenza A virus NS1 protein prevents activation of NF-kappa B and induction of alpha/beta interferon. Journal of Virology 74(24), 11566-11573.

Yao, K. and Vakharia, V.N. (1998) Generation of infectious pancreatic necrosis virus from cloned cDNA. J Virol 72(11), 8913-20.

Yoneyama, M., Kikuchi, M., Matsumoto, K., Imaizumi, T., Miyagishi, M., Taira, K., Foy, E., Loo, Y.-M., Gale, M., Jr, Akira, S., Yonehara, S., Kato, A. and Fujita, T. (2005) Shared and Unique Functions of the DExD/H-Box Helicases RIG-I, MDA5, and LGP2 in Antiviral Innate Immunity. J Immunol 175(5), 2851-2858.

Zhang, Y. and Gui, J. (2004) Molecular characterization and IFN signal pathway analysis of Carassius auratus CaSTAT1 identified from the cultured cells in response to virus infection. Developmental & Comparative Immunology 28(3), 211-227.

Zhang, Y.-B., Wang, Y.-L. and Gui, J.-F. (2007) Identification and characterization of two homologues of interferon-stimulated gene ISG15 in crucian carp. Fish & Shellfish Immunology 23(1), 52-61.

Zhu, R., Zhang, Y.-B., Zhang, Q.-Y. and Gui, J.-F. (2008) Functional Domains and the Antiviral Effect of the Double-Stranded RNA-Dependent Protein Kinase PKR from Paralichthys olivaceus. J. Virol. 82(14), 6889-6901.

Zou, J., Carrington, A., Collet, B., Dijkstra, J.M., Yoshiura, Y., Bols, N. and Secombes, C. (2005) Identification and bioactivities of IFN-gamma in rainbow trout Oncorhynchus mykiss: the first Th1-type cytokine characterized functionally in fish. J Immunol 175(4), 2484-94.

Zou, J., Tafalla, C., Truckle, J. and Secombes, C.J. (2007) Identification of a second group of type I IFNs in fish sheds light on IFN evolution in vertebrates. Journal of Immunology 179(6), 3859-3871.

Zou, J., Yoshiura, Y., Dijkstra, J.M., Sakai, M., Ototake, M. and Secombes, C. (2004) Identification of an interferon gamma homologue in Fugu, Takifugu rubripes. Fish & Shellfish Immunology 17(4), 403-409.

Ørpetveit, I., Gjøen, T., Sindre, H. and Dannevig, B. (2008) Binding of infectious pancreatic necrosis virus (IPNV) to membrane proteins from different fish cell lines. Archives of Virology 153(3), 485-493.

Aaronson, D.S. and Horvath, C.M. (2002) A Road Map for Those Who Don't Know JAK-STAT. Science 296(5573), 1653-1655.

Paper 1

Paper 2

Paper 3