Molecular mechanisms for natural immunity towards infectious salmon anemia virus and infectious pancreatic necrosis virus

Marianne Delima Heisig

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Department of Pharmaceutical Biosciences

School of Pharmacy

Faculty of Mathematics and Natural Sciences

University of Oslo

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Marianne Delima Heisig
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Abstract

When cells are infected with virus, a chain of biochemical processes are initiated to protect the host cell and organism. Some of these processes are effectuated immediately (innate immunity) whilst other processes might need several days before becoming effective (adaptive immunity). An essential part of the innate immune system is the receptors which recognize extracellular or endocytosed/phagocytosed foreign organisms (TLR). In addition there are cytoplasmic receptors (the RLHs RIG-I and MDA-5 or the NLRs) that initiate signaling cascades upon interaction with foreign macromolecules. When these receptors bind foreign molecules, chain-reactions lead to the synthesis and secretion of interferons, certain interleukins, and the activation of different caspases mediating regulated cell-death (apoptosis). The NLRs recognize bacterial components like peptidoglycan containing diaminopimelic-acid or muramyl dipeptide, while the RLHs recognize viral components [1]. When RIG-I bind to viral RNA, down-stream signaling leads to the activation of a mitochondrial antiviral signaling protein (MAVS) that result in increased production of interferon. Activation of virus responsive genes is not always sufficient for protection of salmon against acute infections with the highly pathogenic viruses.

The diseases causing the highest ecological and economic impacts in farmed fish in Europe are caused by RNA-viruses. In this thesis two pathogenic viruses that are the causative agents of two important fish diseases in aquaculture are studied; Infectious Salmon Anemia Virus (ISAV) and Infectious Pancreatic Necrotic Virus (IPNV). Cell lines from Atlantic salmon were infected with these viruses to study the intracellular biochemical changes that are initiated upon infection. In this thesis the importance of the pattern recognition receptor RIG-I and the signaling protein MAVS were studied by transfecting cells with plasmids containing genes coding for these proteins. The effect of overexpressing these proteins on virus replication and CPE were analyzed. In addition to this, the subcellular localization of these proteins was studied by transfecting cells with plasmids coding for variants of RIG-I and MAVS protein fused to Green Fluorescent Protein (GFP). Fluorescence microscopy was utilized to observe and study the effect of virus infection on intracellular localization. Our findings do not conclusively demonstrate that overexpression of RIG-I or MAVS protect the cells from ISAV or IPNV infection.

Table of Contents

Glossary

The following abbreviations are used in the text.

ASK	Atlantic salmon kidney cell line
BSA	Bovine serum albumine
CARDs	Caspase activation and recruitment domain
CARDIF	CARD adaptor inducing IFN-β
CAMs	Cell adhesion molecules
CHSE	Chinook salmon embryo cells
CMV	Cytomegalo virus
CPE	Cytopathic effect
CPM	Cumulative percent mortality
Cq	Quantification cycles
DAPI	4,6-diamino-2-phenylindole, used for nuclear staining
dNTPs	deoxynucleotide triphosphates
DPI	Days post infection
DPT	Days post transfection
dsRNA	Double-stranded RNA
EDTA	Ethylenediaminetetraacetic acid
EF1α	Elongation factor 1 alfa
GFP	Green fluorescent protein
GRP78	Glucose regulated protein 78
HE	Hemagglutinin-esterase
IFN	Interferon
IKK	Inhibitor of NF-κB kinase
Il1β	Interleukin 1β
IPS-1	Interferon-β promotor stimulator 1
IPNV	Infectious pancreatic necrotic virus
IRF-3	Interferon regulatory factor 3
ISAV	Infectious salmon anemia virus
ISG15	Interferon stimulated gene 15
LB-medium	Luria Bertani medium
MAVS	Mithochondrial antiviral signaling protein
MDA-5	Melanoma differentiation-associated gene 5
MCS	Multiple cloning sites
MOI	Multiplicity of infection
NF-κB	Nuclear factor κB
NLRs	NOD-like receptors
NTC	No-template controls
ORF	Open reading frames
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
Poly I:C	Polyriboinosinicpolyribocytidylic acid

PRR	Pathogen recognition receptor
qPCR	quantitative real time polymerase chain reaction
RD	Regulatory/repressor domain
RIG-I	Retinoic acid inducible gene I
RLHs	RIG-like helicases
RLR	RIG-I like receptors
rpm	Rotations per minute
RPS	Relative percent survival
RT-PCR	Reverse transcription polymerase chain reaction
SF2	Superfamily 2
TBK-1	TANK-binding kinase 1
TCID50	Tissue culture infectious dose 50%
TNFα	Tumor necrosis factor α
VISA	Virus-induced signaling adaptor
VRG	Virus responsive genes

1: Introduction

1.1: Atlantic salmon

Atlantic salmon (*Salmo salar* L.) are anadromous fish, i.e. they hatch in fresh water, spend adult life stages in ocean water and then return to fresh water to spawn. Pre-adaption to a life in the sea happens while the salmon is still in fresh water and is termed smoltification. This is a complex transitional life stage which includes both morphological and physiological changes regulated by several endocrine systems [2]. Although Atlantic salmon is prone to infections during all life stages, it has been observed that for example Infectious Pancreatic necrosis virus gives higher mortalities and has the highest incidents in fry and post-smolts, as the salmon's immune system is not yet fully developed [3]. Below, a figure displaying the different life stages for salmon is shown.

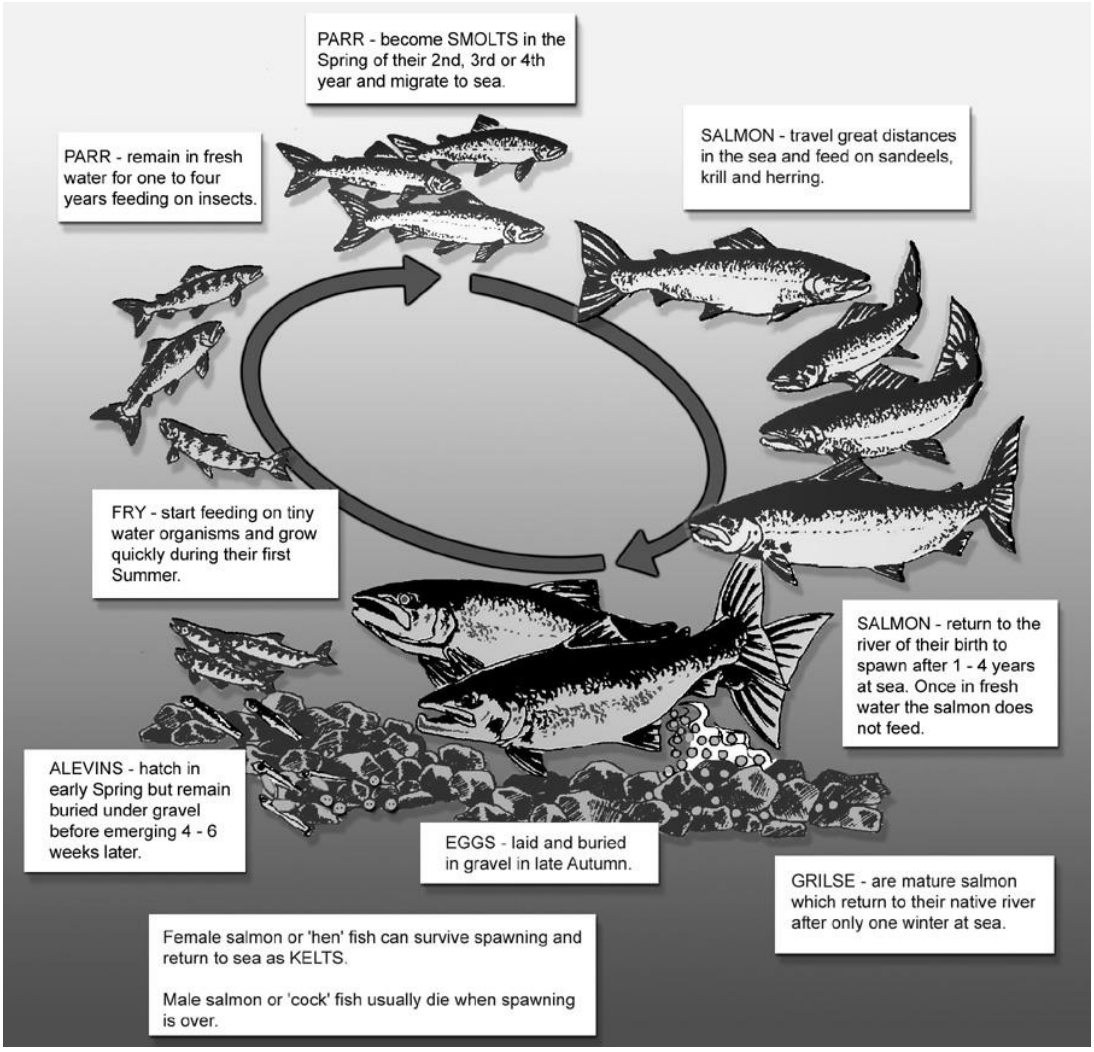

Figure 1: Salmon life stages [4]

Adult salmon lay their eggs in fresh water in special nests in the gravel, during the autumn. The eggs hatch in the spring into alevins. Alevins live off their yolk sacs while still under the gravel. When the yolk sacs have been used up, they have to start feed, and are then called fry. The salmon are known as parr when they are over a year old. The parr change into smolts in their second, third or fourth year and is the process where the salmon is preparing to head out to sea. The adult salmon travels great distances at sea where it feed for one to four years before returning to fresh water to spawn. Adult salmon returning to spawn is known as grilse. After the grilse has spawned they are known as kelts [4].

1.2: *Orthomyxoviridae*

Infectious salmon anemia is an orthomyxovirus infection caused by the aetiological agent Infectious Salmon Anemia Virus, the only virus in the Isavirus genus.

Orthomyxoviridae (*orthos*, Greek for straight, and *myxa*, Greek for mucus) is a family with negative-sense RNA genomes, i.e. a class V in the Baltimore classification. By the molecular biologist's convention, mRNA is defined as a positive (+) strand because it contains immediately translatable information. In the Baltimore classification, a strand of DNA that is of equivalent sequence is also designated a (+) strand. The RNA and DNA complements of (+) strands are designated negative (-) strands [5]. By definition negative-sense RNA genomes cannot be translated directly into protein but first must be copied to make positive-sense mRNA by a viral RNA-dependent RNA polymerase [5]. There are no enzymes in the cell that can produce mRNAs from the RNA genomes of negative-sense RNA viruses. Therefore the virus particles contain virus-encoded, RNA-dependent RNA polymerases to do the task. The negative-sense strand also serves as a template for the synthesis of full-length positive-sense strands, and this newly synthesized antigenome (+) strand is in turn copied to produce negative-sense strand genomes [5]. All viruses with negative sense RNA genomes encode a NP which binds ssRNA. In orthomyxoviruses, the NP is the major structural protein that interacts with the RNA segments, and its primary function is to encapsidate the virus genome to facilitate RNA transcription, replication and packaging [6].

Other members of this family include the Influenza viruses A, B and C, the Thogotovirus and Dhori viruses, but the Isavirus is the only virus in the *orthomyxoviridae* family causing disease in fish that has been described so far. The Influenza viruses of the *Orthomyxoviridae* family depend on higher vertebrates as hosts. In contrast to types B and C, type A is a respiratory pathogen for a wide mammalian host range, including humans, pigs, horses, and

sea mammals, and an enteric virus of many avian species worldwide. In contrast, Thogoto and Dhori viruses replicate in both vertebrate and tick cells and are transmitted by tick bite [7].

1.2.1: Infectious Salmon Anemia Virus (ISAV)

This virus was reported for the first time from an outbreak in Norway in 1984 [8]. ISAV cause 15-100% mortalities in Atlantic salmon, and has emerged as a great concern to aquaculture industry in Europe and Chile imposing great economic losses. Chile lost its second place after Norway as the world's top producer of Atlantic salmon after one outbreak, and the production in 2010 was just 1/10 of Chile's production in 2006 [9]. Since the first outbreak in Norway, ISAV has also been reported from Atlantic salmon in Canada, USA, Scotland and the Faroe Islands, but also in Coho salmon in Chile [8]. In this thesis the ISAV4-strain was used. This strain was kindly provided by Birgit Dannevig at the National Veterinary Institute, Oslo. In a laboratory infection experiment comparing 11 different strains of ISAV, this strain was characterized with relatively high mortality [10]. Little is known of the replication cycle of ISAV, but what we do know is that the optimal replication temperature for ISAV is 15°C and that it does not occur in temperatures above 25°C and that replication and transcription occur in the cell nucleus [6, 7, 9]. This virus shares several genetic, biochemical and morphological properties with Influenza viruses [7].

1.2.2: Virion structure and proteins of ISAV

ISAV is an enveloped, polymorphic single-stranded RNA-virus consisting of 8 RNA segments with negative sense polarity [8, 11]. The viral genome encodes at least ten different proteins, (nine of which are structural) two of them with suggested interferon antagonistic effect. Segment 7 and 8 contains two open reading frames (ORF). The ORF1 of segment 7 encodes a nonstructural protein with interferon antagonistic properties, and the ORF2 of segment 8 encodes an RNA-binding structural protein which also has type I interferon antagonistic properties [11]. One of the most important features with this virus causing virulence, is in the surface protein hemagglutinin-esterase (HE) [3]. HE is one of the most variable of the ISAV genes, exhibiting a length polymorphism close to the 3' end called highly polymorphic region (HPR), and is responsible for receptor-binding [12]. The HE protein-stem length and ISAV virulence has been related although this is not entirely clear [8].

Virions consist mainly of a lipid membranous envelope derived from the plasma membrane of the host cell with two protruding surface glycoproteins (fusion (F) protein and HE) and a matrix protein surrounding a ribonucleoprotein complex [12]. One of these surface

glycoproteins consist of a combination of receptor-binding hemagglutinin with receptor-destroying enzyme activity, and is termed the hemagglutinin-esterase (HE) and is thus responsible for both viral attachment and release. ISAV binds to 5-N-acetyl-4-O-acetylsialic acid and this sugar is also the substrate for the esterase activity in HE [13]. ISAV is closely related to the Influenza A and B viruses in that their RNA is divided into 8 segments, but for the latter viruses, the receptor-binding and receptor-destroying activity is divided between two different proteins; hemagglutinin (which also contain the fusion activity) and neuraminidase. Influenza C viruses encodes a unique multifunctional surface glycoprotein with all three functions (receptor-binding, receptor-destroying and fusion activity) in the same protein; hemagglutinin-esterase-fusion protein (HEF), and their RNA genome are divided into 7 RNA-segments [14]. Also the assignments of the genome segments encoding the proteins in ISAV differ from those of influenza A and B viruses as displayed below where Isavirus and Influenzavirus A are compared.

Table 1: Comparison of genome coded assignments for Isavirus and Influenzavirus A

Genome segment	Gene products[1]	
	Isavirus	Influenzavirus A
1	PB2 (84 kDa)2	PB1 (96 kDa)
2	PB1 (84 kDa)	PB2 (87 kDa)
3	NP (77 kDa)	PA (85 kDa)
4	PA (71 kDa)	HA1 (48 kDa), HA2 (29 kDa)
5	gp50 (47 kDa)	NP (50–60 kDa)
6	HE (42 kDa)	NA (48–63 kDa)
7	p32 (32 kDa), NEP (18 kDa), p11 (11 kDa)	M1(25 kDa), M2 (15 kDa)
8	M1 (24 kDa), NS1 (16 kDa)	NS1(25 kDa), NS2 (12 kDa)

Table 1: [1]Gene products: PB2, PB1 and PA, polymerase; NP, nucleoprotein; HA, hemagglutinin (two subunits in Influenzavirus A); gp50 a fusion protein F (Aspehaug et al.,2005)[15]; HE, hemagglutinin-esterase in Isavirus (Falk et al., 2004) [6]; NA, neuraminidase; M1, matrix protein (Falk et al., 2004); p32, a structural protein of unknown function in Isavirus; M2, ion channel protein; p11, protein of unknown status and function in Isavirus; NS1, non-structural protein 1; NS2, non-structural protein 2 (also known as nuclear export protein, NEP). Table taken from Kibenge et. al. 2004 [16].

1.2.3: Clinical features and pathology of ISAV

In general, infection with ISAV is related mainly to damage of the organs with viral loads highest in the liver, head kidneys, spleen and intestine, before causing death of the salmon [11, 17]. Clinical features include severe anemia, leucopenia, exophthalmia, pale gills, ascites, hemorrhagic liver necrosis, renal interstitial hemorrhage, tubular necrosis and petechia in the viscera [7, 16, 18, 19]. Infected individuals also displays spleen inflammation [17]. Generally, infected Atlantic salmon with ISA appear lethargic and may keep close to the wall of the net pen [11]. The clinical ISA disease can also occur in wild free-ranging Atlantic salmon, but these fish are less susceptible than farmed Atlantic salmon, due either to genetic variation in the two fishes or to stress caused by management practices on salmon farms [16, 20]. ISAV may infect and replicate in sea trout, brown trout, rainbow trout, eels, herring, and Arctic char, resulting in asymptomatic, probably life-long, carriers of the virus [16].

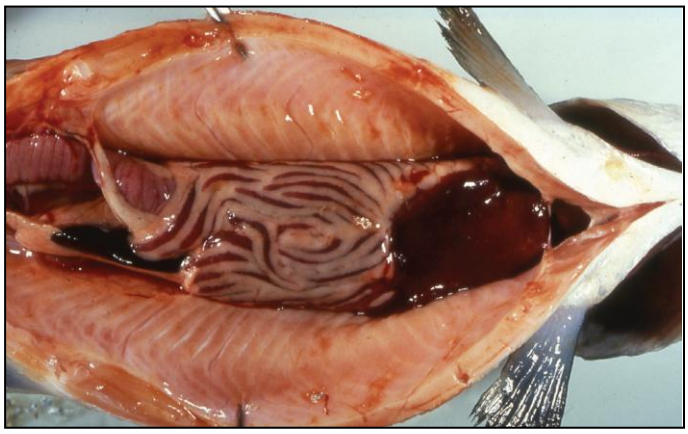

Figure 2: Atlantic salmon with ISA. Dark hemorrhagic liver and intestines in infected salmon [3].

1.2.4: Vaccines against ISAV

Vaccination against ISAV is considered to be a very important strategy in controlling the disease, either by protecting vulnerable stocks or for a ring vaccination program. There is presently no effective available treatments other than destroying all fish in infected farms, avoiding fish movements to and from infected areas [21]. A recent study showed that the nucleotide analogue ribavirin had an inhibitory effect on ISAV replication [9]. Different vaccines against infectious salmon anemia virus have been commercialized. Pharmaq AS, Norway, has the Alpha Jects® vaccine which is an i.p. injection with inactivated monovalent ISAV, currently used in Norway and Chile, but also in Ireland and Finland under the name

Micro-1 ISA, and Novartis Aqua Norway has the Pentium Forte Plus ILA vaccine which is also an i.p. injection vaccine. This vaccine is a multicomponent vaccine against 6 additional diseases. It was not until recently (in 2010) that ISA-vaccination was allowed in Norway. In Canada, an inactivated multivalent i.p. injection vaccine has been commercialized under the name FORTE VI by Aqua Health Ltd., Novartis. In Chile an oral vaccine is available from Centrovet Ltda. This oral vaccine is based on recombinant ISAV protein produced in yeast, mixed with the feed, and can be used on salmon >10 g. However the currently available vaccines do not always confer complete protection, and in addition, vaccines based on inactivated virus must be formulated in oil-water emulsions to induce protection (and are frequently associated with visceral adhesions) [8]. Adjuvants acts like chemical catalysts and increase the response to a vaccine without any specific antigenic effect. Mineral salts, particulate and surfactant based formulation and particulate delivery vehicles like liposomes and virosomes have been used extensively as vaccine adjuvants [22]. The immune response against ISAV after infection or vaccination does not provide full protection against the infection. It has previously been discovered that antibody-mediated uptake and ISAV-replication can occur in macrophage-like fish cell lines which may suggest that Fc (fragment crystallizable) receptor-mediated antibody-dependent enhancement of ISA virus infection might also occur *in vivo,* as the virus in Atlantic salmon targets endothelial cells lining blood vessels and macrophage-like cells [16]. Furthermore the current vaccine delivery by injection is not suitable for dosing large numbers of fish in a cost-effective manner. The relatively low cost per fish and the young age and small size coincident with the high susceptibility of fish to viral diseases at an early age argue against vaccination of individual fish, and highlight the need for means of vaccinating fry or eggs before they leave the hatching shed [16]. If methods of delivering DNA vaccines to first-feeding fry could be developed, these vaccines could be useful in protecting small fish, even before maturation of the specific immune response, early protection being provided by the interferon response [16].

1.3: *Birnaviridae*

Infectious pancreatic necrosis virus (IPNV) is the etiological agent of Infectious pancreatic necrosis. This archetypal aquatic virus belongs to the *Birnaviridae*-family. Characteristics of this family includes viruses with a bi-segmented dsRNA genome that are distinctive, small, nonenveloped and displays a icosahedral capsid with a diameter of 65-70 nm composed of 260 trimers of viral protein 2 (VP2) projecting radially from the capsid [23, 24]. These viruses

exhibit a high degree of antigenic hereogeneity and variation in biological properties such as pathogenicity, host range and temperatures of replication [25].

1.3.1: Infectious pancreatic necrosis virus (IPNV)

Infectious pancreatic necrosis is a serious disease of salmonids and a number of other fish species. IPN is now recognized to be the most important disease in its impact on salmon production in the European Union and in Norway [26]. This could be contributed by the fact that IPNV affects most finfish species in their juvenile stages and causes severe economic losses during juvenile Atlantic salmon release from fresh water to ocean water [26]. Survivors after a outbreak often become persistently infected for a long period. IPNV can be isolated from head kidney from carrier fish, and adherent leukocytes are most likely the sites of persistent infection [27].

1.3.2: Virion structure and proteins of IPNV

IPNV are non-enveloped, and possesses a 60 nm icosahedral structure. Its genome is bi-segmented in RNA segment A and RNA segment B, which in total encodes 5 proteins [28]. RNA segment A has two open reading frames, one long and one short ORF, encoding the 106 kDa polyprotein precursor pVP2-VP4-VP3 and the nonstructural 17 kDa VP5, respectively [24, 29]. VP5 is nonstructural, meaning that it is only detected in infected cells, and not in the purified virion particle itself. During translation in the infected cell, the VP4 of the polyprotein, acts as a viral protease and cleaves its own N and C termini of the polyprotein into pVP2 (62 kDa), and VP3 (31 kDa) [29]. pVP2 is further processed by subsequent cleavages, yielding the mature VP2 which is the major capsid protein and type-specific antigen [29].

VP3 is an internal capsid protein, and its presence together with the mature VP2 are important for correct morphogenesis of icosahedral virions [24]. Since VP2 is the capsid coat protein, this protein displays the humoral antigenic determinants of the virus and is the main target for neutralizing antibodies. This protein is also involved in virus entry, being responsible for receptor recognition [24].

The smaller RNA segment B has a single ORF coding for VP1, which is a minor internal protein of 94kDa, which is the virion-associated RNA polymerase [29]. It has been shown that the VP3 acts as a transcriptional activator by its interaction with VP1. VP3s C-terminal tail interacts with VP1 and removed the structural blockade of the polymerase active site [23].

It is clear that IPNV has evolved mechanisms to overcome the IFN responses. Viral proteins VP4 and VP5 seem the most probable candidates responsible for interfering with the IFN-signaling pathway in salmon [30].

1.3.3: Clinical features and pathology of IPNV

In fresh water, infected fish frequently develops a greatly distended intestine due to accumulation of undigested food. In sea water, after the initial losses, some of the fish that survives fail to grow, and become chronically emaciated and prone to sea louse infection [26]. In Atlantic salmon, the clinical features and pathology are similar to those found in rainbow trout with fry, darker in color, showing in the surface water film or at the outflows, lying on their side and hyperventilating, and also making characteristic shimmering movements, and swirling around their length axis [3, 26]. In young fry, severe necrosis of the pancreatic acinar cells can be observed, together with necrosis of intestinal mucosa, and sometimes some lipid necrosis [26]. In salmon parr, the liver of IPN-infected fish is particularly distinctive. Usually it is swollen, pale yellow to white in color and very friable i.e. easily crumbled or pulverized to the touch [26].

Figure 3: Left: Atlantic salmon parr with acute IPN. The liver is very pale and there are punctuate haemorrhages over the hyperemic pancreatic area [26]. **Right:** Rainbow trout with IPN. The intestine is grossly distended due to undigested food [3]

1.3.4: Vaccines against IPNV

Although there are some commercial vaccines available against IPNV, the disease still continues to be a major problem with frequent outbreaks in farmed salmon fry and post-smolts and in modern aquaculture worldwide. Although some experimental vaccines have been developed so far, only a few have been commercialized, and the protective effects against IPNV demonstrated in laboratory trials are not consistent with field observations. This may be attributed to the lack of effective challenge models for evaluating the efficacy of IPN

vaccines under experimental conditions. Overall, challenge models should produce very high mortality in unvaccinated fish in order to demonstrate the abilities of vaccines to confer protective immunity by reducing mortality in vaccinated fish compared to unvaccinated fish after exposure to challenge virus [31]. Moreover, most vaccine efficacy tests are based on determining the post challenge relative per-cent of survival (RPS) as a measure of protection [31]. Data obtained from these studies do not show the ability of vaccine-induced immune responses to prevent the establishment of infection. Although antibody titers have been reported alongside relative percent survival values in fish, they have not been linked to functional aspects of disease protection. It is not clear whether antibodies produced against IPNV prevent establishment of infection, block dissemination of virus to target organs or reduce replication of virus in target organs in order to prevent establishment of pathological disease [31]. Hence the exact mode of action of vaccine induced protection against IPNV has not been clearly defined. Another confounding factor in IPNV vaccinology, is the ability of the virus to express different variant strains based on amino acid differences within the immunogenic domain of the VP2 capsid [31, 32].

Almost all the salmon farmed in Norway is vaccinated against IPN. The current vaccines probably contribute to a reduced number of outbreaks, but do not prevent infected fish from being asymptomatic carriers of the virus for a long time, thus giving subsequent risk for new outbreaks [3, 28]. One of the reasons why early attempts of vaccination to IPNV had not fulfilled many of their expected beneficial effects, might be because the highest susceptibility to IPNV infection occurs at the early stage when fish are not immunocompetent [8]. There are currently 4 vaccine manufacturers that offer IPNV vaccines. Current commercial vaccines are all oil-adjuvanted i.p. injection vaccines. Pharmaq AS, Aqua Health Novartis and Centrovet produce mono or multi-valent vaccines containing inactivated IPNV, and Intervet Norbio Norwax® Protect-IPN uses a VP2 protein fragment expressed in E. coli [8].

As pointed out by Gomez-Casado et al. [8] that because of the limited efficacy of these vaccines in the field and the presence of tissue adhesion caused by intraperitoneal injection of the oil-adjuvants, alternative vaccines using different antigen delivery systems are needed. Besides, the cost of producing inactivated vaccines is high because it requires cultivation of viruses in bulk followed by inactivation procedures that require proficient safety tests [31].

1.4: RIG and MAVS, the RLR-signaling pathway

The innate immune system is the first line of defense against infectious pathogens and is especially critical for the teleost fish as the adaptive immune response is not as specific as the mammalian system. The innate immune system responds to pathogens by activating host defense mechanisms via interferon and inflammatory cytokine expression. Pathogen associated molecular patterns (PAMPs) are sensed by pattern recognition receptors (PRRs) which are expressed on a variety of different cell types. PAMPs are broadly conserved chemical or structural features present in pathogens but not host cells, thereby serving as alert signals to the innate immune system of the host. PAMPs include viral RNA, cytoplasmic DNA, prokaryotic nucleotide second messengers, and bacterial cell wall components like LPS or flagellar proteins. To date, three families of PRRs, inclunding Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), have been identified for their indispensable role in pathogen recognition and triggering of the antipathogen immune response.

In this thesis the focus was directed towards RIG-I also known as DDX58, which belongs to the RLR-family. This family of receptors is ATP dependent helicases that sense the presence of viral RNA in the cytoplasm of host cells [21]. Members of this family includes RIG-I, MDA5 and LPG2. RIG-I predominantly senses viral RNA containing a 5'triphosphate along with dsRNA regions [21]. This is a perfectly sound target as free 5'-triphosphate RNA end are absent from eukaryotic cytoplasm due to RNA metabolism in the nucleus e.g. mRNA capping. Several ligands has also been identified, like short poly(I:C), and the panhandle structure formed by the 5' and 3' ends of negative-sense RNA genomes [33].

A characteristic feature of RLRs which belong to superfamily 2 (SF2) helicases/ATPases, is an ATPase domain. Besides the characteristic SF2 domain, RIG-I also possess two N-terminally located caspase activation and recruitment domains (CARDs) and a C-terminal RD also termed the C-terminal domain [21]. The CARDs are involved in downstream signaling, while the RD is a major pattern recognition site and senses preferentially dsRNA blunt ends with 5'-triphosphates.

Upon activation by PAMPs, RIG-I associate with the adaptor protein mitochondrial signaling protein MAVS located at the outer mitochondrial membranes. This adaptor molecule working

downstream of MDA-5 and RIG-I, was found in 2005 at almost the same time by four independent labs [34]. MAVS is encoded in the nuclear genome and not in the mitochondrial DNA and is expressed ubiquitously in a variety of tissues and cell types. MAVS also possess an N-terminal CARD [21, 34]. The interaction of the CARDs of RIG-I with MAVS (also called IPS1, VISA, and CARDIF) results in the activation of intracellular signaling cascades, although the mechanism underlying MAVS activation is not understood. When viral RNA is detected it ultimately leads to the activation of the serine/threonine kinases IKKα/β and IKK-i/TBK-1, involving dimer formation of MAVS. IKKα/β then phosphorylates NF-κB, which then translocates to the nucleus to activate its target genes. IKK-i/TBK-1 phosphorylates IRF3 and IRF7, which then dimerizes, translocates to the nucleus and trigger the expression of type I interferons [21]. The type I interferons are cytokines that function in an autocrine and paracrine manner to activate transcription via IFN-stimulated response elements up-stream of ISGs (Interferon stimulated genes). The activation of type I interferons then triggers the expression of ISGs. Secreted type I interferons can activate the JAK/STAT pathway in self and neighboring cells, resulting in the upregulation and production of a large number of antiviral genes, including RIG-I/MDA-5, RNA-dependent protein kinase (PKR), 2',5'-oligoadenylate synthetase (OAS), and major histocompatibility complex (MHC) class I molecules [33]. Still one should keep in mind that fish interferons are not true homologs of mammalian type I interferons, but their expression is activated by a similar regulatory mechanism, which has occurred independently in fish and mammals probably by a process of convergent evolution.

Simply put; upon RNA viral infection, the RNA helicases RIG-I and MDA-5 recognize virus-derived RNA ligands, leading to auto-activation by its drastic conformational changes [34], and they translocate to the outer mitochondrial membrane to interact with MAVS. A previous study revealed that MAVS dimerization through its transmembrane domain is essential for activating NF-κB and IRF-3, and suggested that RIG-I-MAVS interaction induced the MAVS dimers [35].

RIG-I is constitutively expressed, albeit at low levels, and their expression is enhanced by activation of IFN-α/β signaling. In the absence of activators, RIG-I exist in an inactive conformation, which prevents effector access to the N-terminal CARDs and the helicase domain [33]. The transition from the inactive conformation to an active conformation facilitates interactions between the CARDs of RIG-I and MAVS [33].

Many accessory proteins have been identified that play a critical role in mediating the downstream signaling triggered by RLRs, including MAVS, TRIM25 (tripartite motif-containing 25), STING (stimulator of IFN gene, also known as MITA and ERIS) and EYA4 (eyes absent 4) in mammalian cells [36]. MAVS is a critical adaptor for RIG-I, MDA5 and NOD2 signaling, mediating the activation of IRF3 and the subsequent production of type I IFNs. A very recent study by Hou *et. al.* [37] demonstrated that the sequential binding of RIG-I to viral RNA (5'-ppp RNA) and K63 ubiquitin chains induces the formation of large MAVS aggregates, which behave like prions and effectively convert endogenous MAVS into functional aggregates with a potent ability to activate NF-κB and IRF3. The authors suggest that a prion-like conformational switch occurs in MAVS that activates and amplifies antiviral responses and provides a mechanism to mediate robust antiviral responses. Based on their results, the authors proposed a model of MAVS activation that involves the following steps: (1) RIG-I binds to viral RNA through the C-terminal RD domain and the helicase domain; (2) RIG-I hydrolyzes ATP, undergoes a conformational change, and forms a dimer that exposes the N-terminal CARD domains; (3) the CARD domains recruit TRIM25 and other ubiquitination enzymes to synthesize unanchored K63 polyubiquitin chains, which bind to the CARD domains; (4) the ubiquitin-bound CARD domains of RIG-I interact with the CARD domain of MAVS, which is anchored to the mitochondrial outer membrane through its C-terminal TM domain; (5) the CARD domain of MAVS rapidly forms prion-like aggregates, which convert other MAVS molecules into aggregates in a highly processive manner; (6) the large MAVS aggregates interact with cytosolic signaling proteins, such as TRAFs, resulting in the activation of IKK and TBK1 [37].

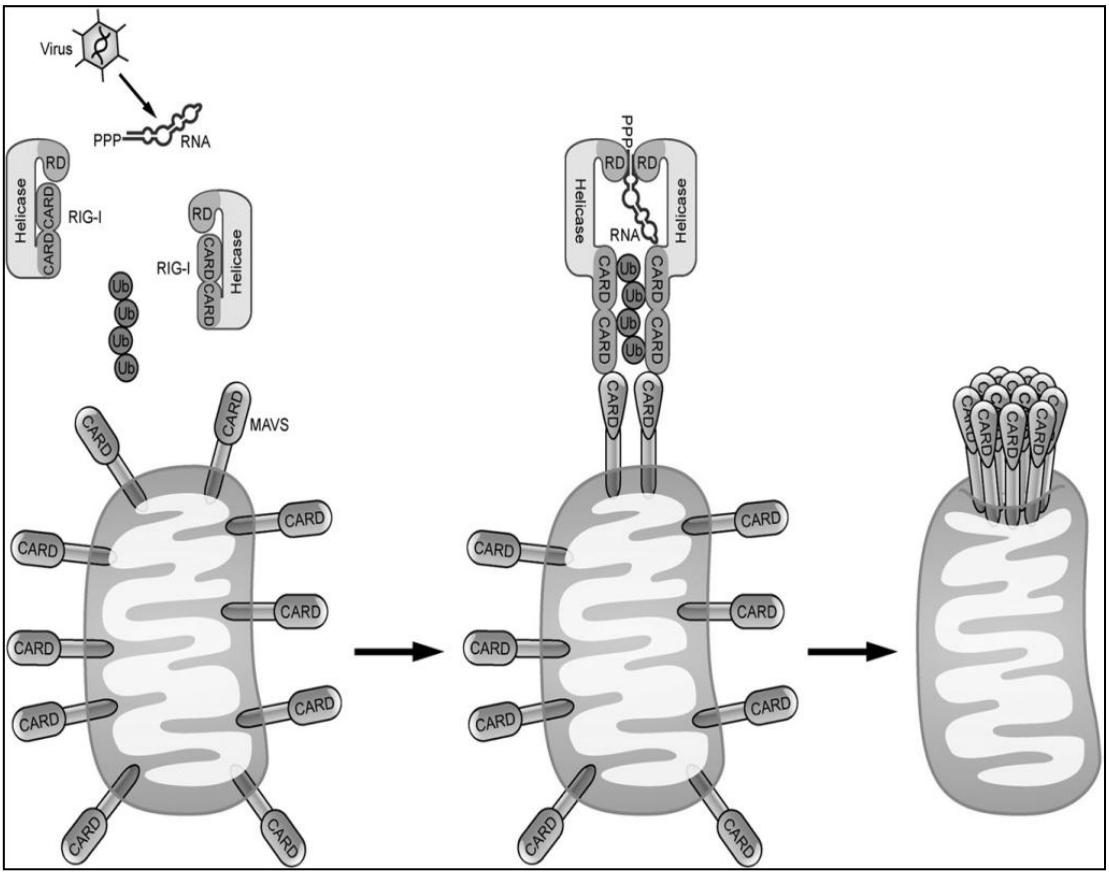

Figure 4: A model of MAVS activation involving a prion-like conformational switch induced by RIG-I [37]

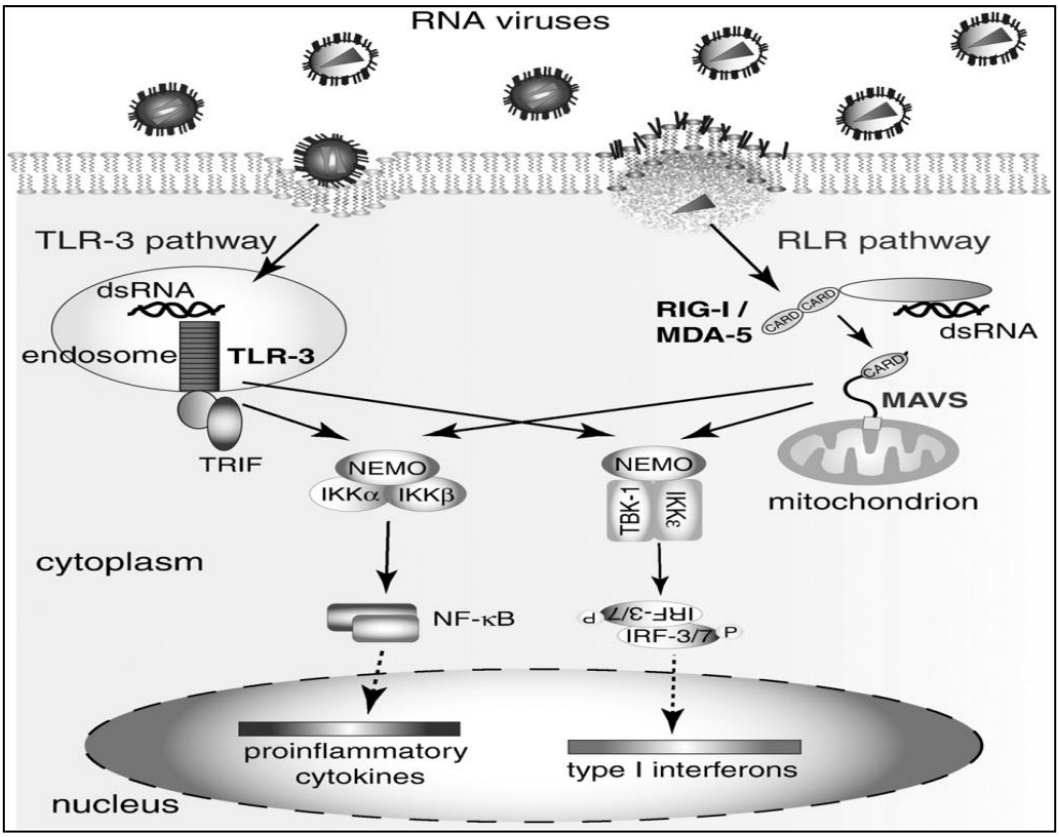

Figure 5: Overview of the RLR pathway [34]

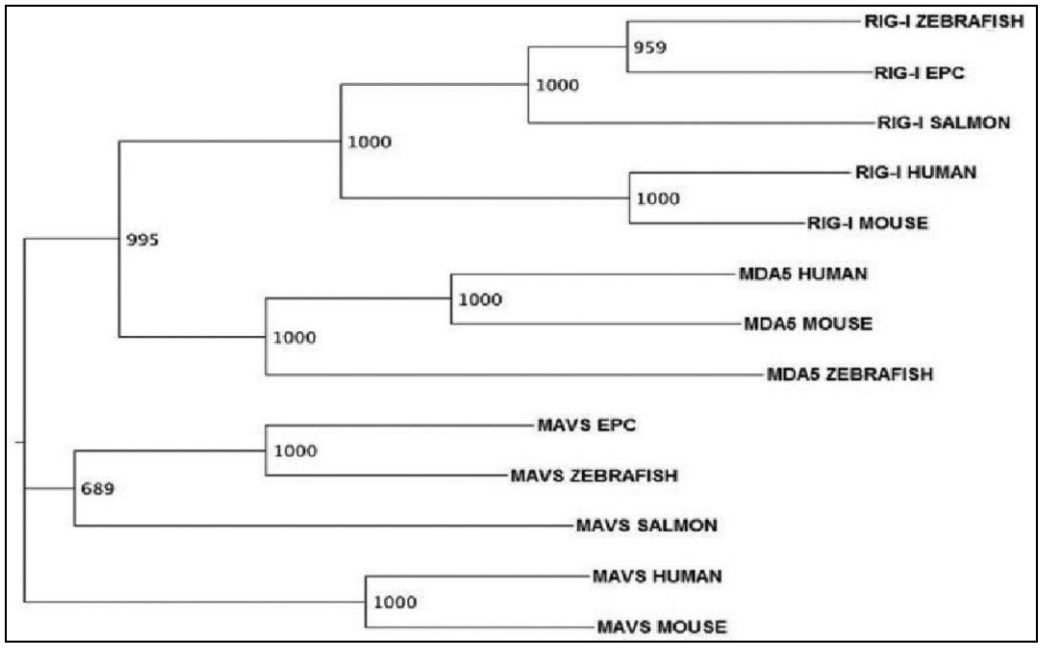

Figure 6: Phylogenetic tree of vertebrate CARDs from RIG-I, MDA5, and MAVS [38]

1.5: Target genes

The relative gene expression of various target genes in ASK and CHSE-214 cells were investigated to see how the relative gene expression would respond to different treatments like transfection with the RIG- or MAVS-plasmid, infection with IPNV or ISAV and poly I:C-stimulation. Several of the genes used as targets in this thesis, were chosen as they are part of the innate immune signaling and response, and would be relevant to examine.

1.5.1: Mx

One of the target genes chosen is Mx (Myxovirus resistance protein). Mx was the first ISG to be studied in fish in relation to virus infection, and has since become a major molecular marker for both viral infection and IFN induction in salmonids and other fish species [39]. Mx is an antiviral protein, but at present time, still very little is known on the inherent ability of teleost Mx protein to prevent viral replication. However, it has been hypothesized that Mx proteins prevent viral replication by preventing intracellular trafficking of viral nucleocapsids [40]. It has previously been shown that over expression of the Mx gene can provide different levels of resistance to IPNV and ISAV in CHSE-cells [41]. Mx is a member of the dynamin superfamily of large GTPase and thus has the ability to self-assemble. The Mx gene is induced by the type I interferon (IFN) the main cytokine regulating innate immune responses directed towards viruses and accumulates in the cytoplasm of IFN-treated cells, associating partly with the endoplasmic reticulum [41].

1.5.2: ISG-15

ISG-15 is one of the IFN-stimulated genes that contain ubiquitinlike domains. This protein is up-regulated early and becomes conjugated to diverse cellular proteins upon IFN stimuli [42]. ISG-15 homologs have been identified in crucian carp, goldfish, Atlantic salmon and Atlantic cod. In Atlantic salmon the 17.3 kDa ISG-15 ortholog increased in expression in response to poly I:C stimulation and ISAV infection. This target gene was chosen in this thesis, as a previous study has shown that immunoprecipitation of ISAV protein from infected TO-cells using an anti-Atlantic salmon ISG-15 antiserum, suggests that ISG-15 has a possible antiviral role in ISAV infection of Atlantic salmon [42].

1.5.3: IFNa

Within the innate immune response the type I interferon system is an essential innate antiviral component that protects fish from some virus infections by inducing an antiviral state in cells. Interferons are widely expressed, inducible and multifunctional cytokines. IFN is secreted by the virus-infected cell and transported by the blood stream to alarm other cells in the body, i.e. the interferons have both an autocrine and a paracrine effect. The antiviral effect is exerted through the binding of IFNa and IFNb to the IFNa/b receptor, which is present on all nucleated cells [40]. Viral infection will inevitably trigger induction of IFNs and ISGs in vertebrates due to the multitude of immune cells and the many viral sensors in host cells. However viruses have developed various mechanisms to antagonize and counteract induction of these genes, making it a race between replication of the virus and the antiviral response of host cells [39].

Unlike higher vertebrates teleosts do not have red bone marrow or lymph nodes as a primary and secondary lymphoid organs; myelopoieses occurs in the teleost head kidney and/or spleen [42]. Type I interferons play a central role in the induction of antiviral responses, as they lead to the activation of many interferon-inducible genes, which influence protein synthesis, growth arrest, and apoptosis. They also enhance dendritic cell maturation, natural killer cell cytotoxicity, and differentiation of virus-specific cytotoxic T lymphocytes, thus providing a bridge between innate and adaptive immune responses [42]. Secretion of IFNs results in the induction of several hundred IFN-stimulated genes (ISGs), some of which encode antiviral proteins such as Mx protein, dsRNA activated protein kinase (PKR) and 2',5'-oligoadenylate synthetase (OAS) [39].

In contrast to the type I IFNs of birds and mammals, which has no introns, the fish IFNs contains 5 exons and 4 introns similar to IFN-γs [40, 42]. In Atlantic salmon there are 11 clusters of IFN genes recently discovered and classified in to three subtypes (a, b and c) of type I interferons, all possessing genes with an intron-exon structure similar to human type II interferon (IFN-λ), although their protein sequences are more similar to IFN-α than IFN-λ. Atlantic salmon IFNa and IFNb are not orthologs of mammalian IFN-α and IFN-β, but appear to utilize similar induction pathways [42]. The Atlantic salmon IFNa is induced in much the same way as mammalian IFN-β (via IRF3 and NF-κB), whereas the IFNb of Atlantic salmon is induced through a similar pathway as IFN-α in mammals (via IRF7) [42]. The mature Atlantic salmon type I IFNs are among the shortest IFNs yet detected [40].

Atlantic salmon IFN-α1has in previous studies shown to induce a potent antiviral activity against infectious pancreatic necrosis virus in cells, but in contrast it did not protect salmon cells against infection by infectious salmon anemia virus, suggesting that ISAV must have developed mechanisms to evade the antiviral effects of IFNs, making IFNa an interesting target gene in this thesis [39].

1.5.4: TNF-α

Tumor necrosis factor-α (TNF-α) is an inflammatory cytokine that produces a local state of inflammation. TNF mediates most of its pro-inflammatory effects by binding to TNF receptor I (TNF-R1). TNF-R1 is a type I transmembrane protein that has cysteine-rich extracellular domains (CRDs) for TNF binding. Its cytoplasmic tail contains a death domain (DD) that recruits the DD-containing adaptor TRADD and kinase RIP1. TRADD facilitates binding of RIP1 to TNF-R1 and recruits TRAF2, which is an adaptor for the ubiquitin ligases cIAP1 and cIAP2 which all contribute to NF-κB and MAPK activation, but the details of how they do so continue to be unraveled [43]. The TNF-α is released mainly by macrophages (but can be produced by other cell types as well) as a result of Toll-like receptor stimulation, and can have both beneficial and harmful consequences [44]. In response to TNF-α, vascular endothelial cells make platelet-activating factor, which triggers blood clotting and blockage of the local blood vessels. This restricts the leakage of plasma from the blood and prevents pathogens from entering the blood and disseminating infection throughout the organism [44]. This cytokine is also involved in apoptosis (programmed cell death). This gene was chosen as a target gene to investigate whether the expression of this inflammatory cytokine is up-regulated or down-regulated during infection with IPNV and ISAV.

1.5.5: IL-1β

Concerning the inflammatory reaction, interleukin 1β (IL1β) is one of the best characterized pro-inflammatory cytokines often used as marker of an activated inflammatory response and was for this reason chosen as a target gene in this thesis. Induction of the cytokine IL-1β by PRRs serves to amplify the inflammatory response because it promotes NF-κB and MAPK activation. Binding of IL1β to IL1R triggers MyD88-dependent signaling [43].

1.5.6: GRP78

Although a protein chain can fold into its correct conformation without outside help, protein folding in a living cell is generally assisted by special proteins called molecular chaperones. These proteins bind to partly folded chains and help them to fold along the most energetically favorable pathway, thus making the folding process more efficient and reliable [45]. The gene encoding GRP78 also known as BiP was chosen as target gene as it has previously been described that the gene encoding glucose-regulated protein 78kDa (GRP78) has been found to be increased in expression following heat shock in rainbow trout cell line RTG-2 indicating its role as a heat shock protein. Viruses rely on the utilization of cellular machinery and resource to complete their life cycle. In this complex process, viruses produce double-stranded RNA intermediates that trigger interferon responses, while viral polypeptides synthesized during infection stimulate ER stress [46]. Several mammalian viruses are also potent inducers of this GRP78 transcript via the ER stress pathway [46] and prolonged ER stress triggers apoptosis (programmed cell death). This molecular chaperone protein is located in the lumen of the endoplasmatic reticulum (ER) and binds newly synthesized proteins as they are translocated into the ER, to aid in correct folding and oligomerization of these proteins. In addition to being a chaperone protein it is also implicated in the immune response. The mRNA is upregulated by bacterial infection in Atlantic salmon, is involved in macrophage development in goldfish, and in differentiation of murine myeloid leukaemia cells into macrophages by the proinflammatory cytokine IL-6 [47]. This protein also possesses an anti-apototic function as it can protect the cell against cell death caused by disturbance of ER homeostasis [48]. As a processing plant for folding and post-translational modifications of proteins, the ER is an essential organelle for viral replication and maturation. In the course of productive infection, a large amount of viral proteins are synthesized in infected cells, where unfolded or misfolded proteins activate the ER stress response [46]. It has been suggested that in mammalian cells the ER chaperone GRP78 works as a master control interacting with three mediators: PKR-like ER kinase (PERK), activating transcription factor 6 (ATF6) and the ER transmembrane protein kinase/endoribonuclease (IRE1) [46]. In response to ER stress, these components function to reduce the levels of new proteins translocated into the ER lumen, to enhance the protein-folding capacity and secretion potential of the ER, and to facilitate transport and degradation of ER-localized proteins [46]. As a resident of the ER chaperone, the expression of GRP78 is upregulated in response to ER stress in the cells. For this reason the GRP78 was chosen as a target gene to investigate whether the IPNV or ISAV would induce ER stress in the cell lines used. Although virus-

mediated apoptosis in ER stress has been recognized, the biological significance of such a process is not well defined [46].

2: Materials

Table 2: Kits	
High-capacity RNA-to-cDNA synthesis kit	Applied Biosystems, Warrington, UK
RNeasy® Mini Kit	Qiagen, MD, USA
RNeasy® Midi Kit	Qiagen, MD, USA
Pierce® BCA protein assay kit	Thermo Fisher Scientific, Rockford, USA
Qiagen Plasmid Midi kit	Qiagen, MD, USA
Amaxa® Cell Line Nucleofector® Kit T	Amaxa Biosystems, Cologne, Germany

Table 3: Reagents	
Ampicillin	Sigma Aldrich, St. Louis, USA
β-mercaptoethanol	Gibco BRL, Uxbridge, UK
Crystal Violet	Sigma Aldrich, St. Louis, USA
Dry milk	Normilk AS, Norway
Ethanol	Arcus, Oslo, Norway
FCS, Fetal calf serum	Gibco-Invitrogen, Paisley, UK
Gentamicin	Cambrex Bio Sciences, Verviers, Belgium
HCl	Merck, Darmstadt, Germany
KCl	Merck, Darmstadt, Germany
KH_2CO_4	Merck, Darmstadt, Germany
Laemmli sample buffer	Bio-Rad Laboratories, CA, USA
Leibowitz L-15 medium	Cambrex Bio Sciences, Verviers, Belgium
L-glutamine	Cambrex Bio Sciences, Verviers, Belgium
Methanol	Arcus, Oslo, Norway
NaCl	Merck, Darmstadt, Germany
Na_2HPO_4	Merck, Darmstadt, Germany
SYBR®Green	Applied Biosystems, Warrington, UK
Trypsin-EDTA	Gibco BRL, Uxbridge, UK

Table 4: Cell lines used in this thesis
ASK-cells
CHSE-214-cells

Table 5: Taxonomic information of the viruses used in this thesis				
Full name	**Family**	**Genus**	**genome**	**Strain**
Infectious Pancreatic Necrosis Virus	Birnaviridae	Aquabirnavirus	dsRNA	V1244, Sp
Infectious Salmon Anemia Virus	Orthomyxoviridae	Isavirus	ssRNA Neg	ISAV4

Table 6: Medium used to maintain both ASK- and CHSE-cells
Leibowitz L-15-medium Supplemented to final concentration with 50 µg/ml gentamicin 4 mM L-glutamine 40 µM β-mercaptoethanol 10% fetal calf serum

Table 7: Crystal violet staining solution 1 litre		
Reagents	**Quantity**	**End concentration**
Crystal violet	0,5 g	0,05 % w/v
37 % formaldehyde	27 ml	1 %
10X PBS	100 ml	1X
Methanol	10 ml	1 %
dH2O	ad 1000ml ≈863 ml	

Table 8: 1X RIPA-buffer
RIPA-buffer is used to lyse cells and tissues 20 mM Tris-HCl (pH 7.5) 150 mM NaCl 1 mM Na_2EDTA 1 mM EGTA 1 % NP-40 1 % sodium deoxycholate 1 tablet of the proteaseinhibitor Complete® from Roche dissolved in 1 ml PBS

Table 9: Composition of the buffers used for plasmid preparation	
Buffer	**Composition**
Buffer P1 (resuspension buffer)	50mM Tris-Cl; pH:8.0, 10mM EDTA, 100µg/ml Rnase A
Buffer P2 (lysis buffer)	200mM NaOH, 1% SDS (w/v)
Buffer P3 (neutralization buffer)	3,0 M potassium acetate, pH 5,5
Buffer QBT (equilibration buffer)	750mM NaCl, 50mM MOPS pH 7,0, 15% isopropanol (v/v), 0,15% Triton X-100 (v/v)
Buffer QC (wash buffer)	1,0mM NaCl, 50mM MOPS pH 7,0, 15% isopropanol (v/v)
Buffer QF (elution buffer)	1,25mM NaCl, 50mM Tris-Cl pH 8,5, 15% isopropanol (v/v)
TE	10mM Tris-Cl pH 8,0, 1mM EDTA

Table 10: Phosphate buffered saline (PBS) 4L	
Reagent	**Amount**
NaCl KCl KH2PO4 Na2HPO4*2H2O MQ	32 g 0,8 g 1,08 g 7,12 g 3950 ml
pH is adjusted to 7,4 with NaOH/HCl	

Table 11: Characteristics of real-time PCR primers used in this thesis

Gene target	Direction	Primer Sequence	Acc. No.	Amplicon size (bp)	E-value
18S rRNA	Forward	TGTGCCGCTAGAGGTGAAATT	AJ427629	61	2
	Reverse	GCAAATGCTTTCGCTTTCG			
EF1α	Forward	CACCACCGGCCATCTGATCTACAA	AF321836	77	1,97
	Reverse	TCAGCAGCCTCCTTCTCGAACTTC			
Mx3	Forward	TGATCGATAAAGTGACTGCATTCA	SSU66477	80	2
	Reverse	TGAGACGAACTCCGCTTTTTCA			
IFNα2	Forward	CCTGCCATGAAACCTGAGAAGA	AY216594	107	2
	Reverse	TTTCCTGATGAGCTCCCATGC			
TNFα	Forward	CGGGTTCAAGCTACAAGGGA	DQ787158	51	2,21
	Reverse	AAGAGCCCAGTGTGTGGGAT			
Il1β	Forward	GGAGAGGTTAAAGGGTGGCG	AY617117	51	2,06
	Reverse	TCCTTGAACTCGGTTCCCAT			
GRP78	Forward	ACGGCATCTTGCGCGTCACA	NM_001141642	254	2
	Reverse	CAGCTTGCCGCCCAGCTTCT			
ISG15	Forward	TGTTAGGTGTCAATGGGAGCAA	AY926456	151	2,03
	Reverse	TGTGTCTGGCCCTTTTCGTT			
ISAV seg7	Forward	GAAATGGACAGAGACGGCGTATCA	AY044132	123	2,05
	Reverse	GCTCAACTCCAGCTCTCTCATTGT			
IPNV ipna	Forward	TACCAATCTGCGGTCTCGAC	U48225	109	2
	Reverse	TGTGTTTCGCACCTCCTCG			
ssMAVS	Forward	AGGAGGTGCTGCACAATGTTGCT	FN178458	95	2
	Reverse	GGCGTCTGGCCGTCGTTGT			
ssRIG-I	Forward	TACTGATCGGGAGAGGACACA	NM_001163699	60	1,83
	Reverse	AGTTCAGCGTCATGCGTGT			

3: Methods

3.1: Propagation of ISAV4 and IPN virus

The same protocol was used for propagation of both ISAV4 and IPNV, but with IPNV fewer days were needed before complete CPE could be observed.

Protocol:

1) ASK-cells were grown in 3-layer incubator flasks in 20°C to 70-80% confluence. The medium was removed, before the ISAV4-inoculate subsequently were poured over the cells together with Leibowitz L-15 medium without serum, to just barely cover the growth surface. The reason for not using medium with serum is that serum may contain glycoproteins that may compete with the ISA-virus for binding to ASK-cells.
2) The incubator flasks with cells and virus inoculate were placed on a "belly-dancer" for 4 hours in 13°C for the virus to adhere to the cells.
3) The virus inoculate was then removed from the cells and stored in -20°C for later use.
4) Leibowitz L-15-medium with above mentioned additives (see Table 6) was transferred back to the incubator flasks.
5) The ASK-cells were then incubated at 13°C until total CPE could be observed when investigating cells in the microscope, i.e. the cells were rounded up and did no longer adhere to the incubator flask. CPE was induced earlier with the IPNV (≈4-6 days) compared to ISAV4 (≈10 days).
6) The media with the virus-killed cells and virus were transferred to plastic centrifuge containers and centrifuged for 20 min at 4000g in 15°C. This first centrifuge step was performed to pellet down the cell debris, so that the supernatant would mostly consist of virus particles. The supernatant was then transferred to an Erlenmeyer flask.
7) The supernatant were divided into 6 centrifuge tubes and centrifuged on a Spin rotor® for 2 hours at 4°C at 29 500 rpm (100 000 x g) to centrifuge down the virus particles. This step was performed twice i.e. for 4 hours in total.
8) The supernatant was then discarded, and the virus pellet was resuspended in 2 ml of PBS by putting the tubes on a shaking-machine in an incubator at 4°C over night.
9) The virus suspension from the 6 corning-tubes was then combined to 1 tube and centrifuged for 2 hours at 29 500 rpm, 4°C to clear out any last cell residual contamination.

3.2: Cell lines

3.2.1: ASK-cells

ASK-cells (Atlantic salmon kidney cells) were kindly provided by B. Krossøy (Department of Fisheries and Marine Biology, University of Bergen, Norway). This cell line was obtained in 1998 from cells taken from the normal kidney of an adult female Atlantic salmon. This cell line is macrophage-like, adherent, grows in a mono layer, and is epithelial in morphology. This cell line has previously been shown to be permissive for propagating both IPNV and ISAV [49].

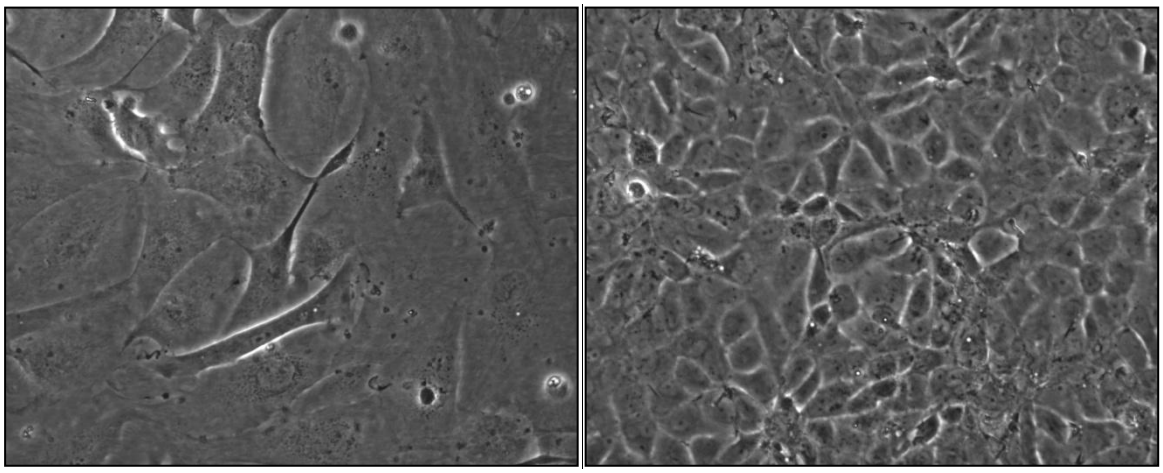

Figure 7: Left: ASK-cells **Right:** CHSE-cells. 40X magnification

3.2.2: CHSE-214 cells

CHSE-cells (Chinook salmon embryo cells) are also an adherent cell line that grows in a mono layer. These cells were obtained from *Oncorhynchus tshawytscha,* Chinook salmon, and are susceptible to a variety of different fish viruses [50]. These cells have a fibroblast-like morphology. This cell line was included in this study due to their higher transfection efficiency than ASK-cells.

3.3: Cell maintenance

Both ASK-cells and CHSE-cells were maintained at 20°C, routinely split once a week and cultured in Leibowitz L-15 medium supplemented as described in **Table 6**. Even though the same media was used for both cell lines, separate flasks were used to prevent cross contamination of the cell lines. For the same reason, the two different cell lines were preferably split on different days if possible. The cell lines were split by using a trypsin-EDTA combination product from Gibco. Cell-cell adhesion in tissues is mediated by a variety

of homotypic interacting glycopeptides called CAMs some of which are calcium dependent e.g. cadherins. These would be sensitive to the chelating effects of EDTA [51].

Protocol used for splitting cells:

1) The medium was removed from the incubator flask and transferred to an Erlenmeyer flask.
2) The cells were washed 3 times with 5 ml PBS.
3) Cells were then trypsinized with either 1 or 2 ml 0.05 % Trypsin-EDTA (1 ml for ASK-cells, 2 ml for CHSE-cells respectively since CHSE-cells were more adherent than ASK-cells), and observed in light microscope to see when the cells were detached. The incubator flask was tapped carefully to make cells successfully detach from the plastic.
4) Some of the medium removed at the start was then transferred back to the incubator flask so that the serum in the culture medium could neutralize any residual activity of the trypsin.
5) The cells split 1:2 i.e. half the volume of cell suspension was either discarded or used for different experiments and the incubator flask was then refilled with medium up to 50 ml.
6) The incubator flasks were then put back for incubation at 20°C.

3.4: Plasmid preparation

Plasmids were prepared with the QIAGEN Plasmid Midi kit where all the buffers were supplied. The buffers used and their composition is described in **Table 9**.

3.4.1: Obtaining single cell colonies with transformed E.coli

Bacterial culture streaking allows bacteria to reproduce on a culture medium in a controlled environment. The bacteria is spread across an agar plate and allowed to incubate at a fitting temperature over a period of time. This method is useful for isolating pure bacterial colonies from a mixed population, in this case a population consisting of transformed and non-transformed E.coli. Transformation is in this thesis defined as cells that has successfully taken up naked DNA and that are expressing the selection marker coded for in the plasmid.

1) 200 ml of LBA-medium was melted in the micro oven until reaching a clear and liquid. 100 ml was transferred into two separate and autoclaved Erlenmeyer flasks.

2) The LBA-medium was allowed to cool down, before 100 µL kanamycin and ampicillin with a concentration of 50 mg/ml, was added to their respective Erlenmeyer flask (final concentration 50µg/ml).
3) The LBA-medium with antibiotics was poured over in petri dishes with approximately 25 ml in each dish.
4) When the agarose dishes were set, E.coli transformed with plasmids containing either RIG-I, MAVS, RIG-GFP og MAVS-GFP-inserts was streaked gently over the dishes with a plastic inoculation loop over the top end of the agar plate moving in a zig-zag horizontal pattern until 1/3 of the plate is covered. The plate is then rotated about 60° and with a new inoculation loop the bacteria are spread from the end of the first streak to a second area with the same pattern. The plate is rotated yet again and bacteria are streaked from the end of the second are to a third area. The GFP-plasmids contain a kanamycin-resistance gene that confers resistance to the transformed bacteria allowing them to grow on the agarose dishes with kanamycin. The plasmids containing the RIG-I and MAVS inserts confers ampicillin-resistance to the transformed E. coli bacteria allowing them to grow on the agarose dishes with ampicillin.
5) The agarose dishes were inverted and then incubated over night at 37°C.

3.4.2: Making the overnight culture for harvesting transformed E.Coli:

6) The following day four 250 ml Erlenmeyer flasks were filled with 100 ml room tempered LB-medium in each flask. Ampicillin was added to the two flasks that were to be incubated with the RIG and MAVS-transformed bacteria, and kanamycin to the two flasks that were to be incubated with the GFP-plasmid transformed bacteria. The antibiotics were added to an end concentration of 30µg/ml.
7) Sterile plastic inoculation loops were used to transfer single colonies to each tube: one with pRIG-I, pRIG-GFP, pMAVS, and p-MAVS-GFP.
8) The Erlenmeyer flasks were incubated at 37°C in a shaking incubator over night.

3.4.3: The plasmid preparation:

9) The following day the bacteria suspension in the Erlenmeyer flasks were transferred to 250 ml centrifuge flasks and pelleted at 6000 x g for 15 minutes at 4°C on a Beckmann centrifuge.
10) The centrifuge flasks were decanted carefully to discard the supernatant. The pellet was then resuspended in 6 ml buffer P1 added LyseBlue until a homogenous resuspension was obtained.
11) 6 ml buffer P2 was then added to the suspension. Inverted 4-6 times and then incubated at room temperature for 5 minutes. The solution should now turn blue.
12) Caps were attached to the nozzle of the Qiafilter midi cartridges.
13) 6 ml of buffer P3 pre chilled to 4°C was added to the cell lysate and mixed by inversion until all the blue color from the LyseBlue in buffer P1 mixed with buffer P2 had disappeared and a white fluffy flocculate has appeared. This indicates that the chromosomal DNA and proteins have efficiently been precipitated, while keeping plasmid DNA in solution.
14) The lysate is then transferred to barrel of the Qiafilter cartridge and the cartridge is then incubated at room temperature for 10 minutes without inserting the plunger.
15) A HiSpeed midi tip column was prepared by adding 4 ml buffer QBT to the column and letting the buffer sieve through by gravitation.
16) The lysate from the Qiafilter cartridge is then filtrated over into the previously prepared HiSpeed midi tip column by removing the cap from the outlet nozzle and inserting the plunger gently.
17) The HiSpeed midi tip column was then washed with 20 ml buffer QC. The flow through was discarded.
18) The DNA was eluated with 5 ml buffer QF.
19) The DNA was then precipitated by mixing it with 3.5 ml room temperature isopropanol and allowing it to incubate at room temperature for 5 minutes to minimize salt precipitation.
20) During the incubation, the plunger from a 20 ml syringe was removed and a QIAprecipitator Midi Module was attached to the outlet nozzle of the syringe.

21) The QIAprecipitator Midi Module was then placed over a waste bottle and the eluate/isopropanol mixture was transferred to the barrel of the syringe. The plunger was gently inserted and the mixture was filtered by exerting constant pressure on the plunger.
22) The QIAprecipitator Midi Module was removed, and the plunger pulled out before the QIAprecipitator Midi Module was reattached. 2 ml of 70% ethanol was added to the syringe. The DNA was washed by inserting the plunger and pressing the ethanol through the QIAprecipitator Midi Module.
23) The QIAprecipitator Midi Module was removed, the plunger pulled out, and the QIAprecipitator Midi Module was reattached. The plunger was reinserted and the membrane was dried by pressing air through the membrane. This step was performed twice.
24) The outlet nozzle of the QIAprecipitator was dried with some absorbent paper to prevent ethanol carryover.
25) The plunger from a new 5 ml syringe was removed and the QIAprecipitator was attached to the outlet nozzle of the syringe. The outlet of the QIAprecipitator Midi Module was held over a 1.5 ml collection tube. 1 ml buffer TE was added to the syringe, and the DNA was eluted by inserting the plunger and exerting constant pressure.
26) The QIAprecipitator was removed, the plunger pulled out, the QIAprecipitator reattached and the eluate from step 25 was transferred back to the syringe and then eluted for the second time into the same collection tube.

3.4.4: PicoDrop measurement of the plasmid preps:

27) The plasmid preps were then measured with a PicoDrop 100™ to determine the concentration and the purity by using the PicoDrop-pipette to draw 2µl of sample up in the disposable UVpette pipette tips that allows UV/Vis measurements to be taken directly through the pipette tip. The 260/280 nm ratio for all preps in this thesis were from 1.90 to 2.20.
28) The plasmid preps were then stored at -20°C until further use.

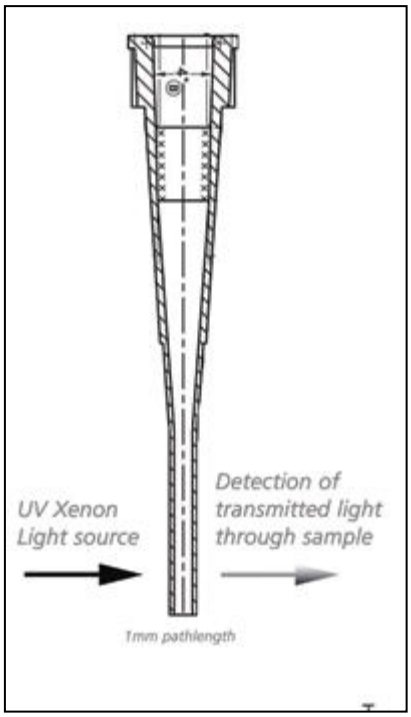

Figure 8: The patented UVpette from PicoDrop

3.5: Plasmids

In this thesis, plasmids with Atlantic salmon (*Salmo Salar*) RIG-I, MAVS, and RIG-I-GFP and MAVS-GFP inserts were used to transfect the cell lines ASK and CHSE-214. The GFP-plasmids are the vectors peGFP-C1 from Clontech, whilst the vectors for the non-GFP plasmids were the pcDNA 1/1 Amp vectors from Invitrogen. Both plasmids contain the cytomegalovirus immediate-early promoter (CMV) driving constitutive expression. These plasmids were kindly provided by Dr. Stéphane Biacchesi, Unité de Virologie et d'Immunologie Moléculaires, INRA, CRJ in France [38]. The full sequence for the plasmids is provided in the appendices.

The discovery of green fluorescent protein revolutionized the study of cell biology, protein expression and subcellular localization. The gene coding for this protein was isolated from the jellyfish *Aequora Victoria* and is a convenient reporter for monitoring transcription and translation, because it is directly visible in living cells without the need for fixation, substrates, or coenzymes [5]. Here the plasmids contain our insert (ssRIG-I or ssMAVS) fused together with the GFP. Many proteins retain their functions when fused with green

fluorescent protein, which permits the study of subcellular location of proteins. In this thesis this fact was exploited to study the subcellular location of RIG-I and MAVS in transfected cells before and after infection. Transfections with the non-GFP plasmids were done to investigate if the over expression of RIG-I or MAVS-protein in the cell would confer any protection of the cells towards the ISA virus or the IPN virus.

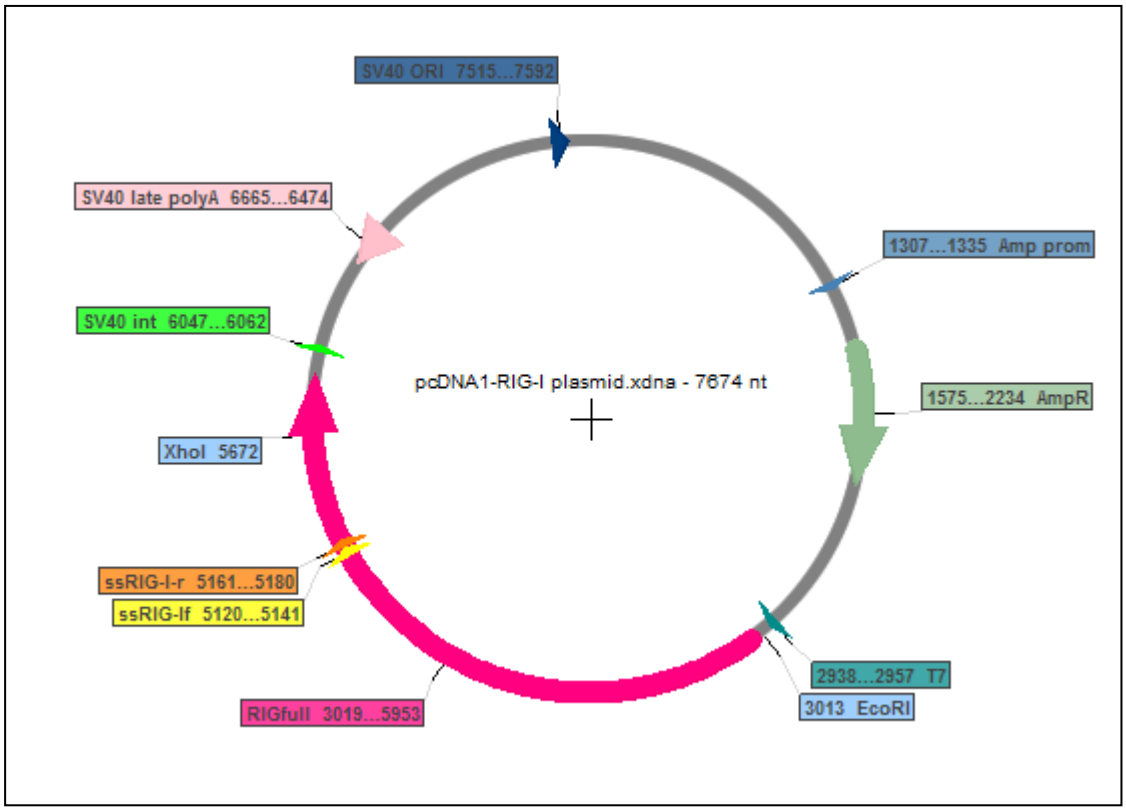

Figure 9: The pcDNA-1-RIG-I plasmid

In Figure 9 above the structure of the full length RIG-I plasmid is shown. The plasmids were visualized by pasting the plasmid sequence into the software SerialCloner version 2.1 (free downloadable software), where the sequence was circularized and the insert and particular sites were tagged. The RIG-I insert is 2934 nucleotides long from position 3019-5953. The pcDNA1.1/Amp vector uses the ampicillin resistance gene as a selective marker allowing us to select only the E. Coli bacterias that were successfully transformed with the plasmid. In addition the replication origin and restriction enzyme sites are also depicted, as well as the position of the RIG-I primers designed and used in this thesis. The pcDNA1.1/Amp plasmid from Invitrogen incorporates the strong cytomegalovirus (CMV) enhancer-promoter sequence for high-level expression, and SV40 and polyoma origins of replication to facilitate episomal replication, in addition to a T7 primer sequence . Use of the CMV promoter has in previous

studies been demonstrated to be the most efficient promoter to drive expression of a plasmid in fish cells [54, 55].

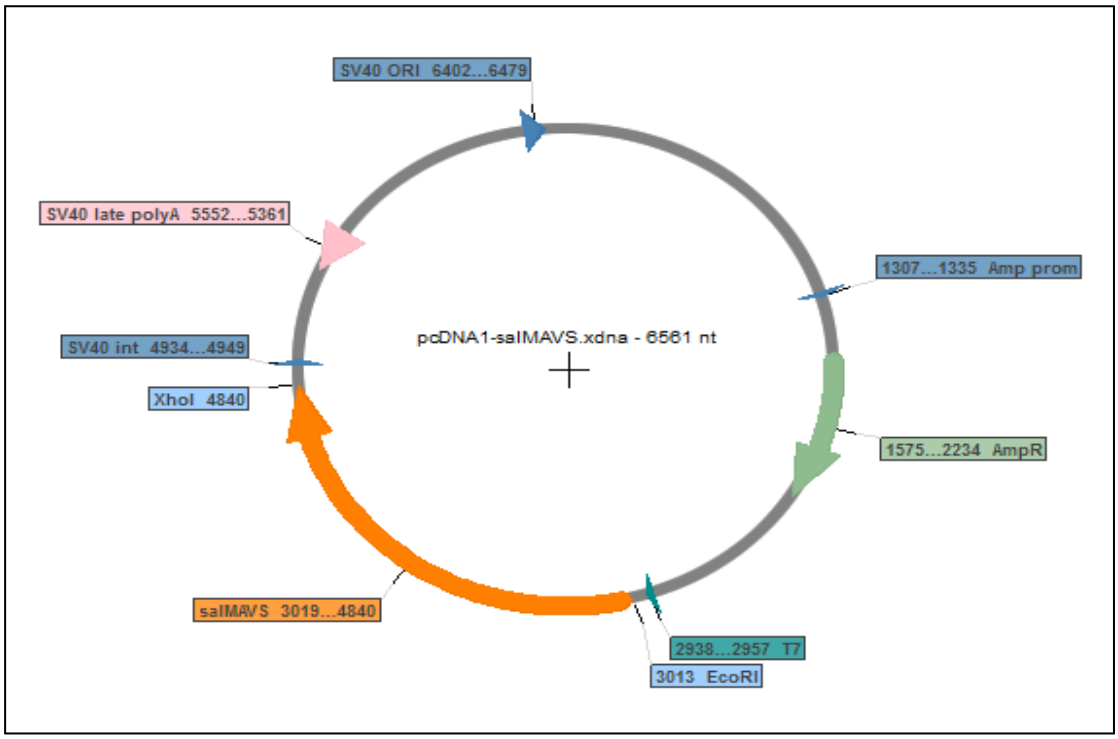

Figure 10: The pcDNA-1/1 Amp plasmid with the ssMAVS-insert

The ssMAVS-insert is 1821 nucleotides long from position 3019-4840.

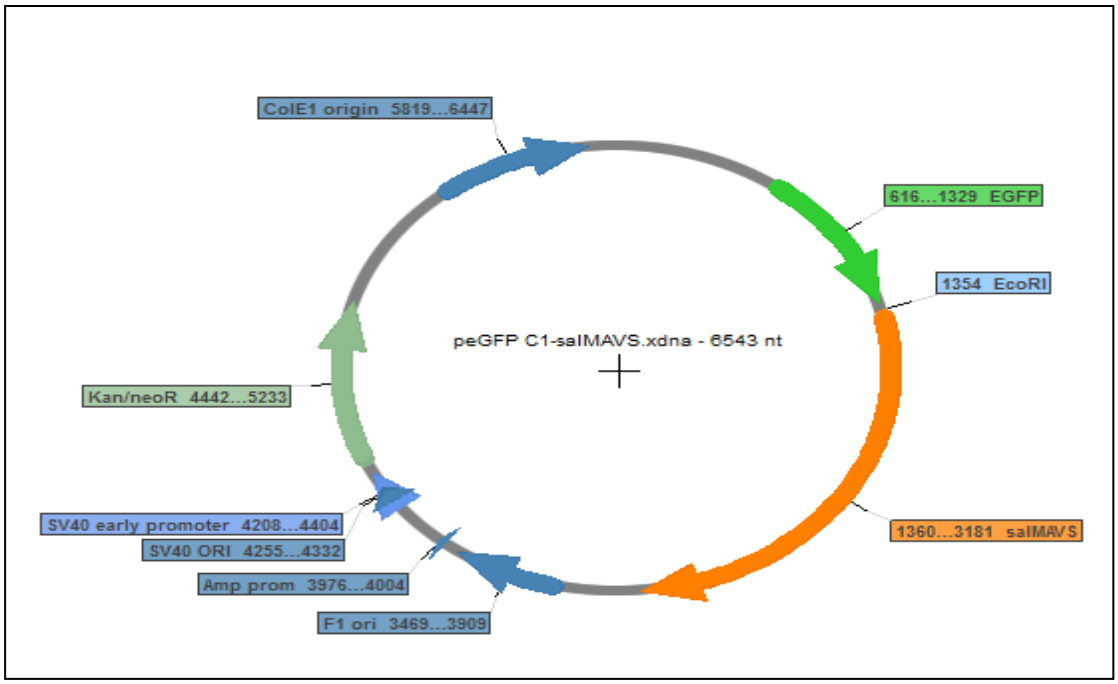

Figure 11: The peGFP C1plasmid with the ssMAVS insert

In Figure 11 seen above, the GFP-insert holds the position from nucleotide 616-1329 and the ssMAVS-insert from position 1360-3181. The peGFP C1 plasmid from Clontech uses Kanamycin/Neomycin resistance as a selective marker for the plasmid.

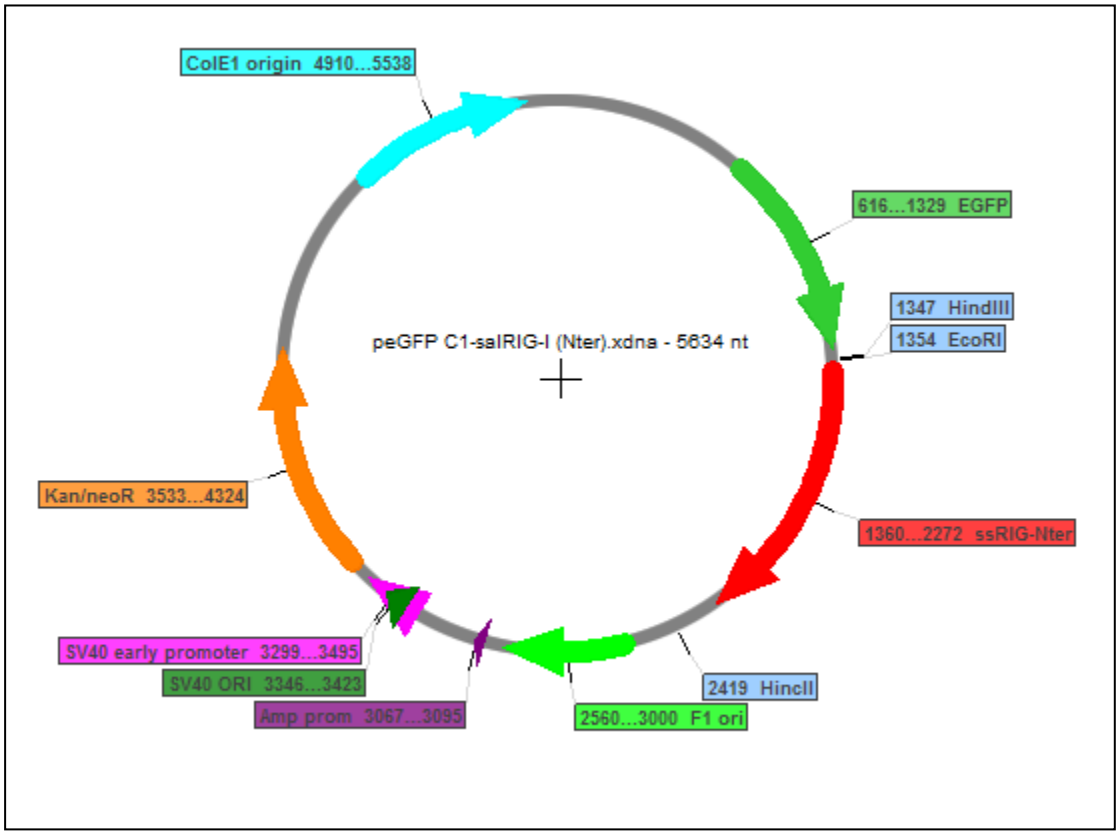

Figure 12: The peGFP C1 plasmid with ssRIG-I (Nter) insert

The GFP-plasmid with the ssRIG-I insert does not contain the full-length ssRIG-I, only the N-terminal end of this gene. This insert is 912 nucleotides long holding the position 1360-2272 in the plasmid.

The peGFP-C1 plasmids from Clontech are under control of a CMV-promoter. peGFP-C1 encodes a red-shifted variant of wild-type GFP which has been optimized for brighter fluorescence and higher expression in mammalian cells and gives an enhanced green fluorescence i.e. eGFP. Genes cloned into the MCS will be expressed as fusions to the C-terminus of eGFP if they are in the same reading frame as eGFP and there are no intervening stop codons .

The overexpression of RIG-I and MAVS in salmon is interesting to study, as the understanding of how these two PRRs can offer protection of the host, offers the possibility of novel vaccines. RIG-I and MAVS are critical determinants required for innate immune

activation in response to viral infection, and agonists that activate these signaling pathways have a potential application as new-generation adjuvants [57]. For use in a licensed vaccine, innate immune agonists would preferably not be an endogenous protein that could potentially induce autoimmune responses. Ideally, these agonists would also not themselves induce adaptive immune response since this would limit repeat usage as well as generate variable results in a population due to attenuated responses in individuals with prior exposure. An RNA-based innate immune agonist would meet these criteria [58].

Since plasmid vectors are already TLR9 and cytoplasmic DNA receptor agonists, incorporation of RLH ligands into the vector backbone of an antigen-expressing DNA vaccine may have targeted immunostimulation application by amplification of type I IFN and cytokine production in transfected cells [58]. RIG-I-activating DNA vaccines may have an application for improving DNA vaccination for various agricultural applications, especially since DNA vaccines have the advantage of ecosafety and being cost effective by using the host cell machinery to produce the antigens thereby avoiding the need for bulk propagation and purification of viral antigens [31, 58].

3.6: RNA isolation

All RNA-isolations performed in this thesis were done with Qiagen RNeasy Mini kit and performed according to the manufacturer's protocol.

1) The samples were obtained by lysis of adherent cells by addition of RTL buffer to the cuture dishes. The samples were stored at -20C° until RNA isolation. The samples were thawed and then mixed on a vortex mixer.
2) 70 % ethanol were added to the samples 1:1, and mixed gently by pipetting up and down.
3) The sample was then transferred to a RNeasy Spin column placed in 2 ml collecting tubes. The tube was centrifuged for 15 seconds at 10 000 rpm. Flow through was discarded.
4) 700µl RW1-buffer was added to the column and centrifuged for 15 seconds at 10 000 rpm. Flow through was discarded.
5) Added 500µl RPE-buffer to the column and centrifuged for 15 seconds at 10 000 rpm. Flow through was discarded.
6) Added another 500 µl of RPE-buffer to the column and centrifuged for 2 minutes at 10 000 rpm. Discarded the flow through.

7) The RNeasy Spin Column was transferred to a new 2 ml collecting tube and centrifuged 10 000 rpm for 1 minute to dry the membrane.
8) The column was transferred to a new 1.5 ml collecting tube, and added 50 µl RNase free water to eluate the RNA. The column was centrifuged for 1 minute at 10 000 rpm.
9) The samples were stored at -20°C for later cDNA-synthesis.

3.7: cDNA synthesis

RNA is prone to cleavage by RNAses leading to degradation. Since cDNA is a thermodynamically more stable product, cDNA was directly synthesized from the harvested RNA-samples prior to q-PCR where gene expression was to be investigated. cDNA-syntheses were performed with High Capacity RNA-to-cDNA pre-dispensed 96-well reaction plates from Applied Biosystems. These 96-well plates are pre-dispensed with 4µl of a 5X master mix which contains a mixture of $MgCl_2$, dNTPs, random primers, oligo(dT) primer and stabilizers, and a reverse transcriptase. With this master mix 5X formula, RNA is allowed to comprise up to 80 % of the reaction mixture. With this method, total RNA isolated from cells in this thesis, were reverse transcribed into first strand cDNA. The oligo(dT) primer anneals at the polyadenylate tail of the mRNA at the 3´-end and then functions as a primer and a start point for the reverse transcriptase, which is a RNA-dependent DNA polymerase isolated from a retrovirus, that transcribes the mRNA from the 3´-end to the 5´-end using all the 4 deoxynucleotide triphosphates producing the first-strand cDNA. These pre-dispensed plates also contain random primers which are short random hexanucleotide primers that are nonspecific and bind to multiple sites along the mRNA.

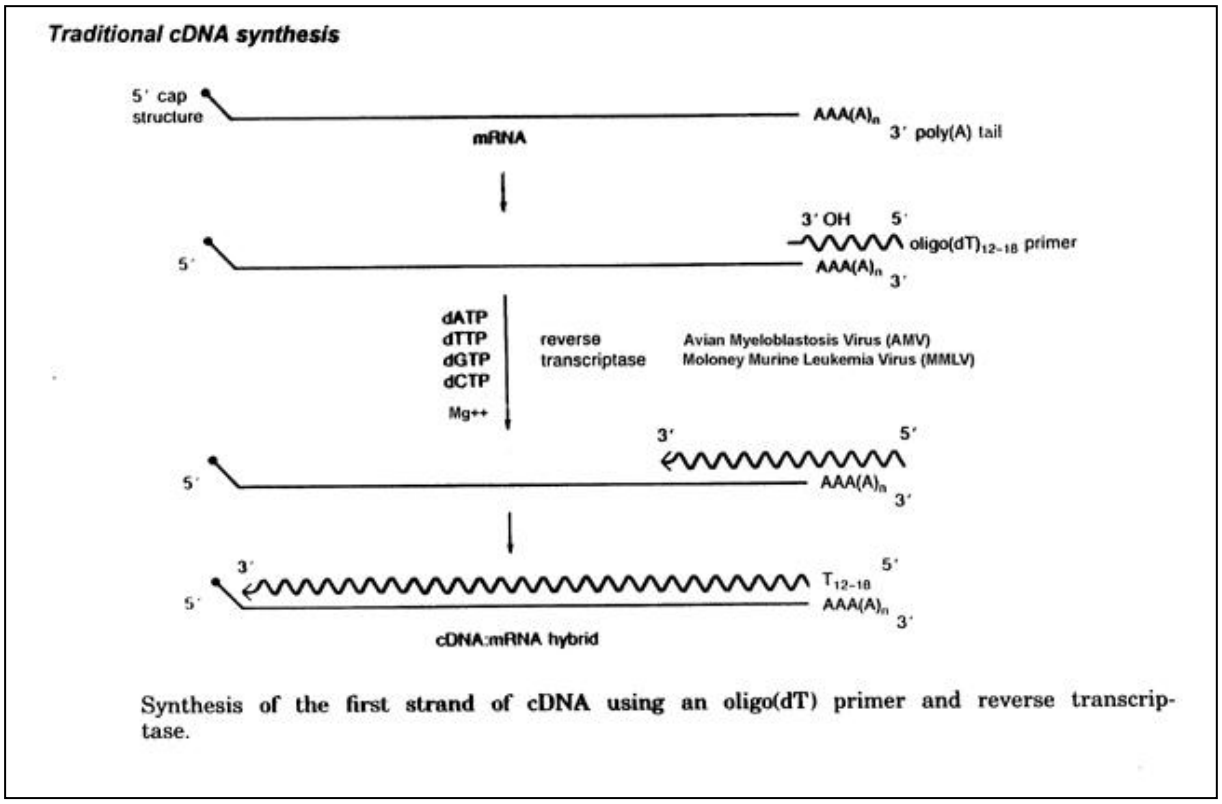

Figure 13: cDNA synthesis

Protocol:

1) RNA-samples were allowed to thaw at room temperature.
2) 16 µl of the RNA-samples were added to each well, and always in duplicates.
3) The plate was then centrifuged at 2000 rpm for 2 minutes at 4°C.
4) After centrifugation, the plate was placed in a thermal cycler from Applied Biosystems and run according to the protocol displayed below in **Table 12.**

Table 12: Cycling protocol for cDNA synthesis	
5 min	25°C
30 min	42°C
5 min	85°C
∞	4°C

3.8: Quantitative real time polymerase chain reaction (Q-PCR)

The impact of the PCR upon molecular biology has been profound and it is now the touchstone for nucleic acid quantification. The reaction is easily performed, and leads to the amplification of specific DNA sequences by an enormous factor making it possible to detect minute amounts of DNA [60]. This is also an invaluable method for expression studies in fish as there is still a limited availability of antibodies. However the standard PCR does not efficiently amplify sequences much longer than about 3kb [60]. Quantitative real-time PCR works in essentially the same manner as end-point PCR, i.e. multiple amplification cycles in which template DNA is initially denatured, followed by annealing of oligonucleotide primers targeting specific sequences, followed by subsequent extension of a complementary strand from each annealed primer by a thermo stable DNA polymerase, resulting in an exponential increase in amplicon numbers during the PCR. Q-PCR-based analyses combine end-point detection PCR with fluorescent detection technologies to record the accumulation of amplicons in "real-time" during each cycle of the PCR amplification [61]. In this thesis the fluorescent report system used was SYBR Green. Originally one first used ethidium bromide, which fluoresce when intercalated into double-stranded DNA, under the development of quantitative PCR, but now SYBR Green is a good alternative as it is not as toxic [60]. SYBR Green I binds in the minor groove of double-stranded DNA via intercalation between adjacent base pairs and when this happens its fluorescence increase over 100-fold [60, 61]. All primers used in this thesis varied in length between 15-25 nucleotides and when designing primers a high GC content was always preferred since this will increase the specificity of the reaction, which is of particular importance for SYBR green assays [61].

Salmon RIG-I primers were designed using the "ProbeFinder" software from ROCHE, **(http://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=uplct_030000)** by pasting in the sequence for ssRIG-I. The salmon RIG-I primers were then ordered from Invitrogen.

In this thesis the transcription activity of the different genes described in **Table 11**, was measured by Q-PCR on samples obtained from ASK-cells that were either IPNV- or ISAV-infected or RIG-I or MAVS-transfected. Q-PCR was also run on samples obtained from ASK-cells that had been stimulated with poly I:C as previously described.

All Q-PCR-experiments followed the same cycling protocol as described in **Table 13** below.

Table 13: Cycling protocol for Q-PCR			
1 cycle pre-incubation	**45 cycles - amplification**	**1 cycle - melting curve**	**1 cycle - cool down**
95 °C 5 min	95 °C 60 °C 72 °C 10 s 30 s 8 s	95 °C 65 °C 97 °C 5 s 1 min	40 °C 30 s

3.9: Q-PCR data analysis

Cycle threshold (C_t) was set automatically by ROCHE Lightcycler® 480 Real Time PCR system software for each PCR reaction. High C_t-values were discarded manually. To calculate the relative expression of the target genes, C_t-values of target genes and reference genes were analyzed using the Relative Expression Software Tool (REST©) [62] which calculates the relative expression with the ΔΔCt-method. Q-PCR assays were performed on a LC480 96-well plate real time PCR machine (Roche, Burgess Hill, UK). The sequences of the primers are presented in **Table 11.** Relative expression of the target genes were normalized against the relative expression of the two reference genes elongation factor 1α and 18S. REST also compares groups using a *pairwise fixed reallocation randomization test* to calculate differences between groups (2000 randomizations). $P < 0.05$ was considered significant.

3.10: Transfection of cells

In this thesis, transient cell transfection was done with two different methods; the one being a liposome-transfection and the other one a transfection performed using electroporation of the cells to transfer the plasmids used in this thesis into the ASK- and CHSE-cells. Because fish cells have longer cell cycles than mammalian cells and lower optimal temperatures for growth, transfection of fish cells generally offers a lower transfection efficiency compared to mammalian cells [54], but there has been a lot of research into optimizing the transfection efficiency of fish cell lines [54, 55].

Liposome transfection was generally a more gentle way to transfect the cells, i.e. fewer cells died after the transfection, but with this method a lower transfection ratio was obtained compared with the method using electroporation. The electroporation gave a higher transfection ratio, but also stressed the cells a lot more, and gave fewer viable cells. In contrast to lipid-based transfection, where DNA is transferred into the cytoplasm and can only

enter the nucleus during mitosis, when the nuclear envelope is disintegrated, nucleofection is supposed to deliver DNA into the nucleus independent of cell cycle [55].

Liposome transfections were performed using the X-tremeGene HP DNA transfection reagent from Roche. This is a low toxicity transfection method which is simple to perform and applicable to many cell types that are difficult to transfect by other means. These liposomes are phospholipid vesicles, which spontaneously form stable complexes with DNA, and create lipoplexes. These interact with the cell membrane resulting in DNA uptake by endocytosis [60].

Transfections performed with electroporations were performed with the Amaxa™ Nucleofector™ technology using the Amaxa® Cell Line Nucleofector® Kit T. The principle behind this method is the creations of nanometer-sized pores in the cell membrane, by exposing cells to a brief pulse of electricity. DNA enters the cell through these pores, and is transported to the nucleus of the cell. This technique was first applied to animal cells by Neumann & Wong (1982) who successfully introduced plasmid DNA into mouse fibroblasts [60]. Larger numbers of cells may be required than for other transfection methods because in many cases, the most efficient electroporation occurs when there is up to 50% cell death.

3.10.1: Liposome transfection protocol:

1) Cells were grown to 50-70% confluence.
2) The X-tremeGene HP DNA transfection reagent was warmed to room temperature, and then vortexed gently before use.
3) An appropriate volume of Leibowitz L-15 culture medium without added serum was added to an eppendorf tube, and plasmid and transfection reagent was added in the ratio 1:1, 1µg DNA to 1µl transfection reagent as this was found to be the most optimal mix ratio in this thesis. Pipetted gently to mix completely.
4) The mixture was incubated at room temperature for 30 minutes for complex formation.
5) The complexes were distributed evenly to cells in complete culture medium.
6) The cells were incubated for 24-72 hours in 20°C before different experiments on the cells were carried out.

3.10.2: Electroporation transfection protocol:

1) Cells were trypsinized and counted with a hemocytometer. The appropriate number of cells was centrifuged at 1000 rpm for 5 minutes. Always used double the amount of cells needed since 50% of the cells usually die.
2) The cell pellet was resuspended in Nucleofector solution T. 100 µl per transfection.
3) In eppendorf tubes plasmid DNA and cell suspension was mixed:
   20µl plasmid DNA (≈1µg DNA) + 100µl cell suspension
4) The contents of the eppendorf tubes were transferred to cuvettes and run in the Amaxa Nucleofector machine with the T-027 settings.
5) The contents in the cuvettes were transferred to their incubator flask or well plates, previously filled with culture medium, using a Pasteur pipette.
6) Cells were incubated for 24-72 hours at 20°C. The medium was changed after 24 hours to remove dead cells.

3.11: Infection trials

CHSE- and ASK-cells were infected for different experiments with IPNV and ISAV following different setups. All infection trials were performed using adding virus at an MOI of 1.

Infection of ASK-cells in 24-well plates:

	1	3	5	7	9	Control/Mock infected
A						
B						
C						
D						

← ISAV

←IPNV

Cells were split with 30 000 cells in each well in 1 ml culture medium and incubated for 24 hours at 20°C. Every other day, cells were infected with 100µl virus inoculation ≈MOI of 1, and pictures were taken of the cells for a time series to observe how the infection proceeded over time, and how infection with IPNV or ISAV differed from each other.

3.12: Fluorescence microscopy

3.12.1: Studying the subcellular localization of RIG-I-GFP and MAVS-GFP

CHSE-cells were amaxa-transfected with RIG-GFP plasmids and MAVS-GFP plasmids before splitting them to 8-chamber plates with 30 000 cells per chamber in 500µl culture medium. The cells were allowed to incubate for 24 hours at 20°C before staining with DAPI, Mitotracker Red CMXROs, Lysotracker Red DND-99 and Alexa Fluor® 568 Phalloidin.

Molecular probes	DAPI	Mitotracker Red + DAPI	Lysotracker Red	568 phalloidin + DAPI
	MAVS-GFP	MAVS-GFP	MAVS-GFP	MAVS-GFP
	RIG-GFP	RIG-GFP	RIG-GFP	RIG-GFP

Staining with DAPI:

DAPI is a popular nuclear counterstain for use in multicolor fluorescent techniques. Its blue fluorescence stands out in vivid contrast to green, yellow, or red fluorescent probes of other structures. DAPI preferentially stains dsDNA.

The culture medium was carefully removed from the chamber. The cells were then washed twice with 500µl room temperature PBS prior to fixating cells with freshly prepared 3.7% PFA for 10 minutes at room temperature. Cells were then washed again twice with 500µl PBS. 300µl 300nM DAPI in PBS was then added to each chamber and left to incubate with the cells for 5 minutes before washing with PBS. A little PBS was added to the chamber before microscopy to keep the cells from drying.

Staining with MitoTracker® Red:

The MitoTracker® probes passively diffuse across the plasma membrane and accumulate in active mitochondria. Once their mitochondria are labeled, the cells can be treated with an aldehyde-based fixative for samples that need fixation to allow further processing of the sample.

The culture medium was carefully removed. Mitotracker Red diluted in Leibowitz L-15 medium without serum to 500nM was added to the chambers ≈400µl per chamber. The cells

were left for 30 minutes before the liquid was removed. The cells were washed twice with 500µl PBS, before the cells were fixated for 15 minutes with 3.7% PFA.

Staining with Lysotracker Red DND-99:

Weakly basic amines selectively accumulate in cellular compartments with low internal pH and can be used to investigate the biosynthesis and pathogenesis of lysosomes. The LysoTracker® probes are fluorescent acidotropic probes for labeling and tracking acidic organelles in live cells. These probes have several important features, including high selectivity for acidic organelles and effective labeling of live cells at nanomolar concentrations. The LysoTracker® probes, which consist of a fluorophore linked to a weak base that is only partially protonated at neutral pH, are freely permeant to cell membranes and typically concentrate in acid organelles. Their mechanism of retention has not been firmly established but is likely to involve protonation and retention in the membranes of the organelles with acidic pH.

The dye was diluted to a working solution of 75nM in PBS. 500µl was added to each chamber and left to incubate with the cells for at least 30 minutes in room temperature. Then the liquid was removed before adding 100 µl PBS to keep the cells from drying out during microscopy.

Staining with Alexa Fluor® 568 phalloidin:

Phallotoxins were originally isolated from the deadly *Amanita phalloides* mushroom and are bicyclic peptides. The phalloidin used in this thesis, is a derivative of this toxin but differs with two amino acid residues. Fluorescent phallotoxins stain F-actin at nanomolar concentrations and are extremely water soluble, thus providing convenient probes for labeling F-actin in cells.

The cells were washed twice with 500µl PBS. Cells were then fixated with 3.7% PFA for 10 minutes. Cells were then washed again two-three times with PBS. Then 0.1% triton X-100 in PBS was added to the chamber and left to incubate with the cells for 3-5 minutes. After the extraction the cells were then washed with PBS three times. 0.25µl Alexa Fluor® 568 phalloidin was then added to each chamber in 200µl PBS with added BSA (0.5mg/ml). Cells were left to incubate with this solution for 20 minutes at room temperature. The cells were washed twice with PBS, before adding 100 µl PBS to keep the cells from drying out during microscopy.

3.12.2: Immunofluorescence of virusinfected cells

ASK-cells were split to 8-chamber plates with approximately 30 000 cells per chamber in 500µl culture medium. 4 plates were used: Two of the plates contained transfected cells, and two plates were infected with IPNV and ISAV. See setup below. Cells were amaxa-transfected as described earlier, and cells were infected with a MOI of 1. Virus was visualized by using fluorescent secondary antibodies against the primary antibodies against the viruses; K-267 against IPNV and Falk 3H6F8 directed against ISAV HE. The latter antibody was kindly provided by Dr. K. Falk at The Norwegian Veterinary Institute, Oslo, Norway [63].

0 dpi	1 dpi	3 dpi	5 dpi
RIG-GFP	RIG-GFP + IPNV	RIG-GFP + IPNV	RIG-GFP + IPNV
MAVS-GFP	MAVS-GFP + IPNV	MAVS-GFP + IPNV	MAVS-GFP + IPNV

0 dpi	1 dpi	3 dpi	5 dpi
RIG-GFP	RIG-GFP + ISAV	RIG-GFP + ISAV	RIG-GFP + ISAV
MAVS-GFP	MAVS-GFP + ISAV	MAVS-GFP + ISAV	MAVS-GFP + ISAV

0 dpi	1 dpi	3 dpi	5 dpi
Untreated cells	IPNV	IPNV	IPNV
Untreated cells	IPNV	IPNV	IPNV

0 dpi	1 dpi	3 dpi	5 dpi
Untreated cells	ISAV	ISAV	ISAV
Untreated cells	ISAV	ISAV	ISAV

3.13: Poly I:C stimulation of cells

Protocol:

1) The ASK-cells were to be seeded in 24-well plates with approximately 30 000 cells per well in 1 ml culture medium.

$$mL\ of\ cell\ suspension\ needed = \frac{30\ 000\ cells\ x\ 24\ wells}{X\ cells\ per\ ml\ of\ cell\ suspension}$$

2) The needed volume of cell suspension was then transferred to a 50 ml corning tube and diluted with the needed volume of culture medium.

3) 1 ml of this diluted cell suspension is then transferred to each well and the plate was then incubated over night at 20°C.
4) One day after splitting the ASK-cells to 24-well plates (30 000 cells/well) the cells were stimulated with 10µg/ml final concentration of poly I:C in each of the 20 wells. The 4 wells that were left over were mock stimulated with 100µl of Leibowitz L-15 culture medium.
5) The 24-well plate was incubated at 20°C and samples were to be harvested at day 1, 2, 3, 4 and 5 days post stimulation.
6) When harvesting samples, the medium was removed from the well, and the cells were lysed in 350µl of RLT-buffer. The plate was allowed to incubate at room temperature with the RLT-buffer for approximately 2 minutes.
7) The cell lysates was transferred to eppendorf tubes and stored at -20°C until RNA-isolation.

A B C D	Day 1	Day 2	Day 3	Day 4	Day 5	Mock stimulated

After RNA-isolation and subsequent cDNA-synthesis of the samples, the samples were then subjected to q-PCR analysis.

3.14: Protein measurements

Prior to performing the western blots, the samples from harvested cell lysates were measured for protein content with the kit from Pierce® BCA Protein Assay. This method was introduced in 1985 by Paul K. Smith, et al, and has since then become the most popular method for colorimetric quantitation of total protein. Bicinchonic Acid (BCA) Protein assay makes use of the reduction of Cu^{2+} to Cu^{1+} when peptides containing three or more amino acid residues i.e. proteins chelate with Cu^{2+} in an alkaline environment. This is termed the biuret reaction. The Cu^{1+} produced in this first step, then reacts with two molecules of BCA producing a purple colored reaction product [64]. This BCA/Copper Complex exhibits a strong linear absorbance at 562 nm with increasing protein concentrations.

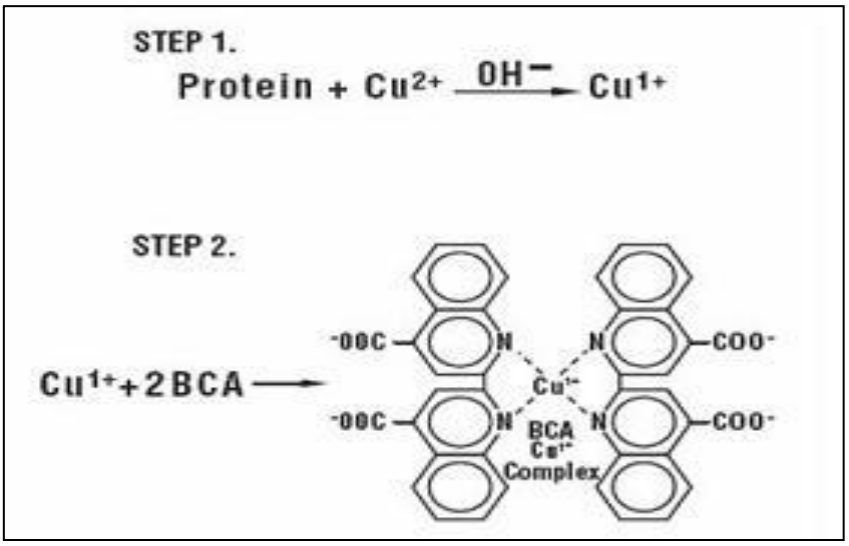

Figure 14: Reaction schematic for the BCA protein assay **[64]**

Protocol:

1) The cell lysates in RIPA-buffer with complete, was thawed on a rotator in 4°C for 30 minutes.
2) The reagents in the Pierce® BCA Protein Assay Kit were then mixed in this ratio: 50 parts reagent A with 1 part reagent B.
3) The BSA-standard (0,5mg/ml) was diluted 1:1 with RIPA-buffer.
4) 200µ of the working solution (reagent A mixed with reagent B) was added per well in a 96-well plate. Sample and standard where then added in duplicates accordingly to the table shown below:

Table 14: Plate setup used for BCA assays

	1	2	3	4	…12
A	Blank	10 µl standard	5 µl sample 3		
B	Blank	10 µl standard	5 µl sample 3		
C	1 µl standard	20 µl standard	5 µl sample 4		
D	1 µl standard	20 µl standard	5 µl sample 4		
E	2,5 µl standard	5 µl sample 1	5 µl sample 5		
F	2,5 µl standard	5 µl sample 1	5 µl sample 5		
G	5 µl standard	5 µl sample 2			
H	5 µl standard	5 µl sample 2			

5) The 96-well plate was covered with plastic film/foil and then incubated at 37°C for 30 minutes.
6) The plate was then read in a HTS 7000 Plus Bio Assay Reader, and the absorbance was measured at 570 nm.
7) The protein content was calculated by using the standard curve (OD for µg/well) and interpolating the protein content in each sample by the µl added. This was automatically done with the HTSoft 2.0 software.

3.15: Western blots

The term "western" blotting (Burnette 1981), refers to a procedure which involves the transfer of electrophoresed protein bands from a polyacrylamide gel on to a membrane of nitrocellulose or nylon, to which they bind strongly. Western blotting is a powerful and commonly used tool to identify and quantify a specific protein in a complex mixture. The bound proteins are then available for analysis by a variety of specific protein-ligand interactions, like in this case where antibodies are used to detect specific antigens [60]. Primary antibodies from rabbits and mice were used to probe the target, and are themselves detected in a sandwich reaction using a secondary antibody raised in goat and donkey directed towards the primary antibody. The sandwich method may also give a substantial increase in sensitivity, owing to the multivalent binding of antibody molecules [60].

3.15.1: Sample preparation

CHSE-cells were split to two 6-well plates with approximately 150 000 cells in 3 ml culture medium per well (see **Table 6).** The cells were incubated overnight in 20°C prior to infection. The following day the cells were infected with a MOI of 1 -> 150µl of virus inoculate per well (one plate with ISAV, the other with IPNV), except for the control wells that was left uninfected. After adding the virus, the plates were incubated over night at 13°C. Samples were harvested every other day with 300µl RIPA-buffer with complete, by removing the medium, adding the RIPA-buffer and allowing the buffer to work for 30 minutes in 4°C with "shaking" every five minutes. Then a cell scraper was utilized to detach the cells before the cell lysate was transferred to eppendorf tubes and stored at -20°C till further use.

Table 15:The plate setup for westernblott samples		
Control, uninfected	1 dpi	3 dpi
5 dpi	7 dpi	9 dpi

3.15.2: Electrophoresis

When an electric field is applied to a solution containing protein molecules, the molecules will migrate in a direction and at a speed that reflects its size and net charge. This forms the basis of the technique called electrophoresis. Individual polypeptide chains form a complex with negatively charged molecules of sodium dodecyl sulfate (SDS) and therefore migrate as a negatively charged SDS-protein complex through the polyacrylamide gel. Before applying the samples to the wells of the gel, the samples are mixed with Laemmli sample buffer that amongst others contains the reducing agent β-mercaptoethanol to break any –S-S- linkages in or between proteins. Under these conditions, proteins migrate at a rate that reflects their molecular weight.

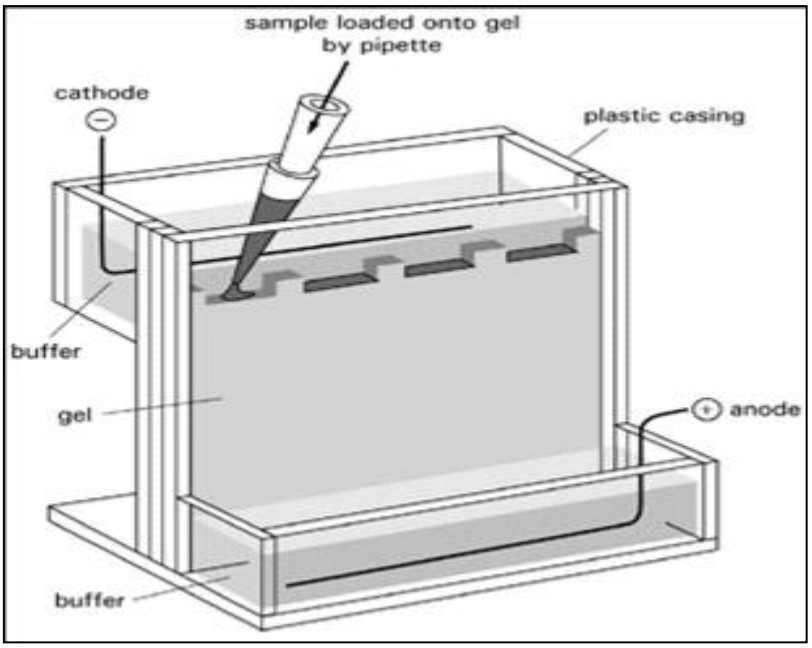

Figure 15: Gel electrophoresis **[45]**

The electrophoresis was performed either with precast graded 4-20% Precise™ protein gels from Thermo Scientific and run with a tris-HEPES-SDS buffer, or 10% polyacrylamide gels cast before run with Elfo-buffer. The gel was made 1.5 mm thick with 10 wells using the Bio-Rad Mini PROTEAN casting chamber:

1) A glass front and back plate was rinsed with ethanol before being assembled in the Mini-PROTEAN casting chamber.
2) The upper and lower gel buffer was prepared. See **Table 16** below.

3) The lower gel was mixed in a 50 ml corning tube. See **Table 17.** TEMED was added just before use, since this is the component that makes the gel set. Then the solution was poured into the gap between the glass plates.
4) Isopropanol was added to the gap to make the lower gel set with a straight surface. The gel was allowed to set for 15 minutes.
5) The isopropanol was gently poured off, and the components of the stacking gel was mixed together and poured into the gap. The plastic "comb" was then placed into the stacking gel to make the wells, and the gel set in 15 minutes.
6) When the gel had set, the plastic "comb" was gently removed, and the gels were assembled into a container with the frontside in. The container was then filled with ELFO-buffer (**Table 18)**
7) The samples were thawed, and mixed with Laemmli sample buffer in the ratio 1:1 (**Table 19**). Then the proteins in the samples were denatured by heating the samples up to 95°C for 5 minutes.
8) 20µl sample was added to each well, and the gel was run for 1-2 hours at 100V, 190 mA.

Table 16: Buffer solutions used for gel casting

Solutions	Reagents	Amount
Lower gel buffer	Tris-base SDS 20 % MQ	18,17 g 2 ml ad 100 ml
	pH was adjusted to 8.8 with NaOH/HCl	
Upper gel buffer	Tris-base SDS 20 % MQ	6,06g 2 ml ad 100 ml
	pH was adjusted to 6.8 with NaOH/HCl	

Table 17: Gel solutions for western blot		
Solutions	Reagents	Amount
Lower gel	MQ Lower gel buffer Acrylamide TEMED APS 10 %	6,0 ml 3,6 ml 4,8 ml 7,2 µl 72 µl
Upper gel/Stacking gel	MQ Upper gel buffer Acrylamide TEMED APS 10 %	4,8 ml 2,0 ml 1,2 ml 8,0 µl 40 µl

Table 18:ELFO-buffer 1 litre		
Reagent		Amount
Distilled water 10X TGS	 Tris-Glycine-SDS-buffer Tris 25 med mer Glycine 192 mM, pH 8.3 SDS 0,1 % w/v	900 ml 100 ml

Table 19: Laemmli sample buffer	
Reagent	Amount
Laemmli sample buffer MQ β-mercaptoethanol	2,5 ml 2,25 ml 250 µl

3.15.3: Blotting

1) The gels were gently released from the glass plates, and put in a sandwich between nitrocellulose membrane, filter paper and padding in a frame. Since the proteins are covered in SDS with a negative charge, the proteins will move from the negative pole to the positive pole.
2) The frames were placed in a container, the container was filled with blotting buffer, see **Table 20**, and a little stirring magnet and an ice block was placed into the chamber.
3) The membranes were blotted for 2 hours in 4°C on a magnet stirrer at 60V, 400mA.

Table 20: Blotting buffer 1 litre	
Reagent	Amount
TG-buffer	100 ml
Distilled water	700 ml
Methanol	200 ml

3.15.4: Ponceau-staining of the membrane

The effectiveness of the protein transfer from the gel to the membrane was verified by staining the nitrocellulose membrane in Ponceau-solution. This solution has a bright red color and is water soluble making it easy to wash away after use. The protein bands on the membrane will be colored bright red due to the high sensitivity of the ponceau solution.

1) The membranes are carefully removed from the "sandwich" and placed in a small plastic container with the protein side up.
2) The ponceau solution was poured over, and the membranes were allowed to incubate in the solution until the protein bands were visible.
3) The membranes were then cut to fitting sizes and washed in distilled water before they were placed in 50 ml corning tubes with the protein side inwards.

3.15.5: Blocking

To reduce nonspecific binding, the membrane is washed with dry milk so that the milk proteins can coat the nitrocellulose leaving only the target proteins the available place to bind for the antibodies.

1) The membranes were washed with 5 ml TBS with 5 % dry milk 2 times for ten minutes on a rotator, and then for the third time for 1 hour at room temperature.

3.15.6: Adding primary antibodies

The traditional method for making antibodies of desired specificity is to immunize animals with the appropriate antigen and then prepare antisera from their blood. The specificity and quality of such antisera is highly dependent upon the purity of the immunizing antigen preparation because antibodies will be made against all the foreign components it contains.

1) Primary antibodies were added to the tubes 1:1000, i.e. 5 µl per tube in the TBS-dry milk solution.
2) The tubes were left on a rotator over night in 4°C.

Table 21: Primary antibodies used for westernblotts		
Virus	Name of antibody	Obtained from
	anti-actin	Rabbit
ISAV	anti 6AD/9Z (HE)	Rabbit
IPNV	anti-VP3	Mouse

3.15.7: Adding secondary antibodies

1) The liquid is removed, and the membranes are washed 3 times with 5 ml TBS 0.05 %, 10 minutes each time.
2) Adds 5 ml TBS with dry milk 5% to the tubes, and adds 1 µl secondary antibodies to the tubes. The tubes that had primary antibodies from rabbit, was added the secondary antibodies donkey anti-rabbit IgG-HRP, while the tube with the mouse-obtained primary antibody was added the secondary antibody goat anti-mouse IgG-HRP, from Santa Cruz Biotechnology.
3) The tubes are left on the rotator in room temperature for 1-3 hours.
4) The membranes are washed 3 times with 5 ml TBS, 10 minutes each time.

3.15.8: Detection

Chemiluminescent substrates are popular because they offer several advantages over other detection methods and yields the greatest sensitivity of any available detection method. Super Signal® West Femto Maximum Sensitivity Substrate Kit from Pierce Thermo Fisher Scientific, is an ultra-sensitive chemiluminescent substrate system for western blotting with horseradish peroxidase (HRP) enzyme. Chemiluminescent substrates differ from other substrates in that the light detected is a transient product of the reaction that is only present while the enzyme-substrate reaction is occurring. This is in contrast to substrates that produce a stable, colored product; these colored precipitates remain on the membrane after the enzyme-substrate reaction has terminated.

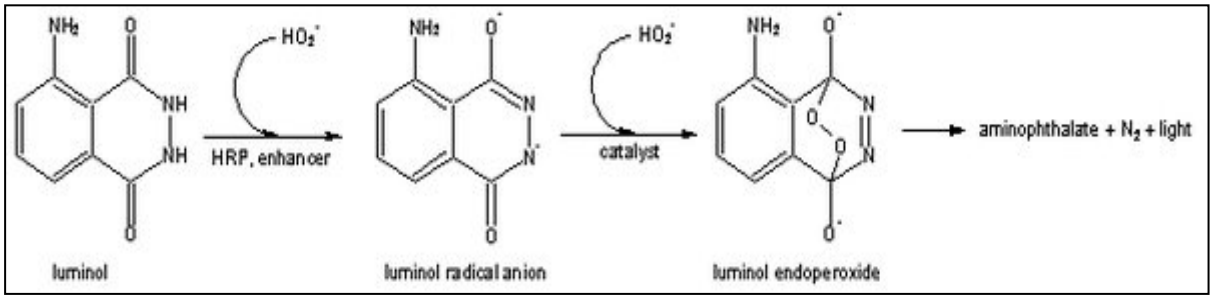

Figure 16: Chemiluminescent reaction of luminol

1) The membranes are placed in appropriate plastic pockets.
2) The reagents of the Super Signal® West Femto Maximum Sensitivity Substrate kit were mixed 1:1, reagent A with reagent B, to make the working solution.
3) 500µl is pipetted over each membrane, and the protein bands are detected in a Chemi Genius^2 Bio Imaging System from Syngene, using the software GeneSnap.

3.16: $TCID_{50}$ titration of virus

This method is an end point dilution assay that quantifies the amount of virus required to produce CPE in 50% of the inoculated cells. CHSE-cells were used as host cells, and serial dilutions of either IPNV or ISAV were added. The samples was harvested from control, MAVS- and RIG-transfected cells, to investigate if the transfection and overexpression of MAVS and RIG gave any protective effects against the virus i.e. gave any reduction of $TCID_{50}$/ml compared to non-transfected cells infected with the virus. This method is often used to get a picture of how much virus is needed to produce CPE in 50 % of cells, since knowing the actual amount of virus particles in an inoculate isn't always indicative of how many virus particles that are actually able to infect a cell. In fact many virus particles are defective and unable of replication, and only a fraction these virus particles have been assembled correctly upon release from the host cell.

Protocol used to obtain samples for later $TCID_{50}$ titration:

1) CHSE-214-cells were split, centrifuged and resuspended in Nucleofector® solution T as previously described.
2) Three nunclon cell flasks (25 cm^2) were filled with 5 ml Leibowitz L-15-medium as described in **Table 6**.
3) In eppendorf tubes plasmids and cell suspension was added:
   MAVS (30,72ng/ µl) 20 µl + 100 µl cell suspension
   RIG (64,77ng/ µl) 20 µl + 100 µl cell suspension
   Control (max-GFP 0,5µg/ µl) 4 µl + 100 µl cell suspension.
4) The contents of each eppendorf tube were transferred to 3 separate cuvettes and run in the Amaxa Nucleofector® on program T-027.

5) The contents of each cuvette were then transferred to the 25 cm^2 cell flasks previously filled with medium by using a Pasteur pipette.
6) The cells were then allowed to incubate for 4 days in 20°C.
7) 4 days after the transfection, a 20 ml corning tube was filled with 1 ml of either ISAV or IPNV inoculate and 15 ml Leibowitz L-15 medium.
8) The media was removed from the cell flasks, and 5 ml of the diluted virus inoculate was transferred to each flask. The cells were then allowed to incubate with the virus for 1 hour for the virus to adhere to the cells.
9) The virus inoculate was removed and 5 ml new Leibowitz L-15 medium was filled in each cell flask.
10) The cells were then incubated at 13°C

500µl of the media was then harvested every other day (day 1, 3, 5 and 7 post infection) and stored at -20°C until $TCID_{50}$-titration.

Protocol for $TCID_{50}$ titration:

1) CHSE-214-cells were washed three times with 5 ml PBS, before subsequent detachment with 0.05 % trypsin-EDTA.
2) To neutralize the residual activity of the trypsin, Leibowitz L-15 medium with serum was added to 10 ml in total.
3) The cells were then counted in a hemocytometer. For 96-well plates approximately 10 000 cells should be seeded in each well, and 0,2 ml medium/well is sufficient.

$$\frac{10\,000\ cells \times 96\ wells \times 2\ plates}{cells\ per\ ml\ in\ cell\ suspension} = volume\ of\ cell\ suspension\ needed$$

$$(0{,}2\ ml\ medium\ per\ well \times 96\ wells \times 2\ plates) - \ volume\ of\ cell\ suspension = volume\ of\ medium\ needed$$

4) The calculated amount of medium and cell suspension was mixed and 200 µl of this diluted cell suspension was pipetted over each well.
5) The 96-well plates were incubated at 20°C over night.
6) The harvested virus samples were thawed at room temperature one day after the cells were split to 96-well plates. The samples used were the samples harvested at day 3 and

5 for both IPNV- and ISAV-infected cells that were transfected with max-GFP, MAVS or RIG. 100µl of the virus sample were then diluted with 900µl of L-15 medium 1:10, and serial diluted 1:10 six times so that we ended up with 6 dilution series.

7) 100 µl of virus inoculate 1: 10 was added to wells 1 A-H, 100 µl of virus inoculate was added to wells 2 A-H and so on…
8) The 96 well plates were covered with plastic cover tape sheets and incubated at 13°C for the appropriate days depending on which virus used. IPNV usually needed ca 7 days, but the ISAV needed several weeks. This was the general plate setup:

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A												
B												
C												
D	1E-0,1	1E-0,2	1E-0,3	1E-0,4	1E-0,5	1E-0,6	1E-0,1	1E-0,2	1E-0,3	1E-0,4	1E-0,5	1E-0,6
E												
F												
G												
H												

Day 3: control, MAVS or RIG Day 5: control, MAVS or RIG

9) After the incubation, the medium was removed from the 96-well plates by decanting.
10) 200µl of the crystal violet solution described in **Table 7** was added to each well with a multi pipette.
11) The plates were left to incubate for 20 min at room temperature before the staining solution was gently removed.
12) The plates were then submerged in a bucket of water with the tap running to gently wash the plates.
13) The plates were then allowed to air dry before the positive wells (wells where CPE was apparent) i.e. the wells that weren't stained with the crystal violet was counted and the plates were photographed on a light box.

14) The $TCID_{50}$ and the $TCID_{50}$/ml was then calculated by using Reed and Muench $TCID_{50}$-calculator, an infectivity calculator that can be easily found on the internet [65].

In addition to this, the crystal violet dye was redissolved in 100µl of 1% SDS solution, and the optical density (OD) was read on the 96-well plate measuring the absorbance at 590 nm using the HTS 7000 Plus Bio Assay Reader. This was done to aid in reducing the subjectivity of determining whether a well is positive or negative by eye [41].

4: Results

4.1: Viral infection of cells with IPNV and ISAV

Infection of CHSE-cells with these two pathogenic viruses produced different effects. Generally IPNV produced a more prominent CPE, with the cells round up and detaching after 4-5 days (Figure 17). With ISAV the cells did not produce a prominent CPE even when the cells were incubated with the virus for several weeks. The cells however became vacuolarized although they did not detach from the surface as seen in Figure 18.

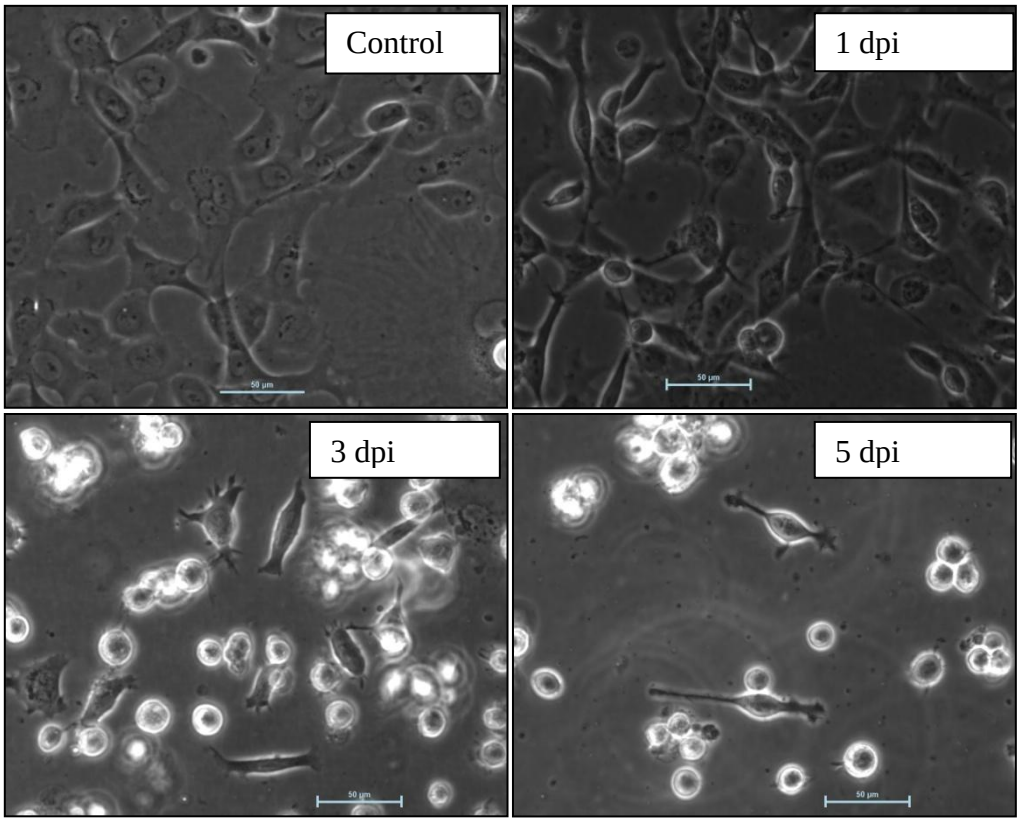

Figure 17: IPNV infection in CHSE cells. 40X

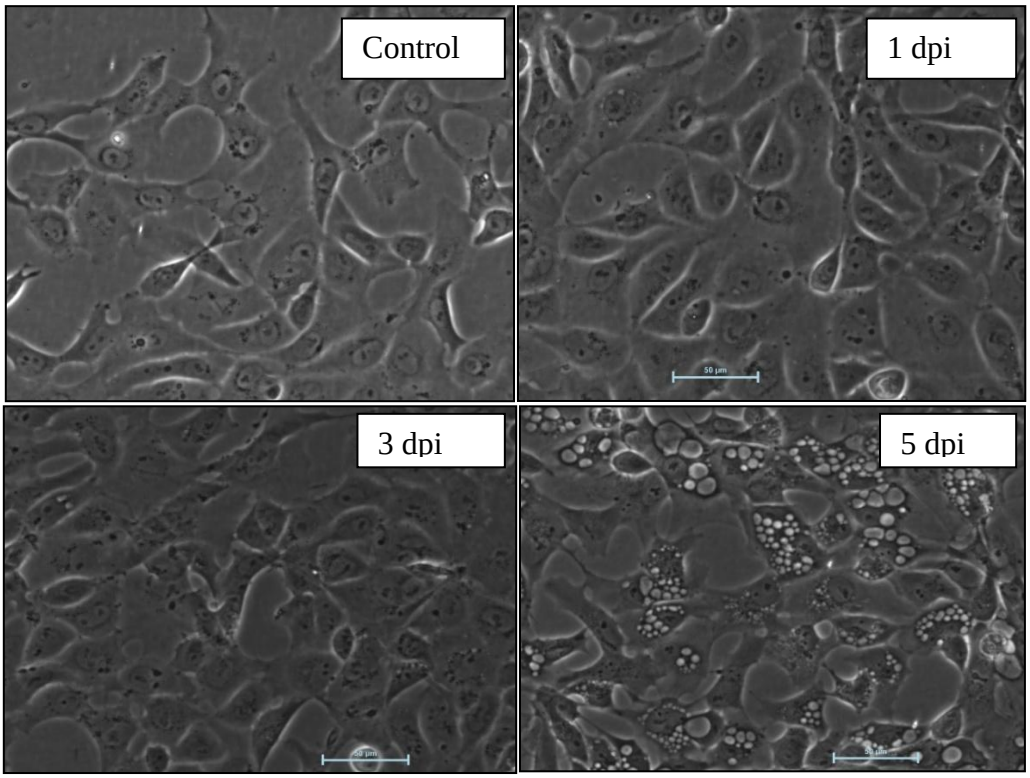

Figure 18: ISAV infection in CHSE-cells. 40X

4.2: Viral replication of ISAV and IPNV

To monitor viral replication of ISAV and IPNV in ASK-cells by means of increased mRNA levels of ISAV segment 7 and IPNV segment A, Q-PCR was performed on cDNA from total RNA samples from infected ASK-cells at 1, 3, 5, 7, and 9 days post infection. The ASK-cells had been infected with an MOI of 1. The figure presented below shows the relative expression of segment 7 in ASK-cells calculated relative to day 1 post infection. Relative expression levels were calculated in REST® for days 3-9 post infection. The level of mRNA for ISAV segment 7 increased steadily throughout the sampling period and reached a level more than 210 times the level at day 1 post infection. This demonstrated that ASK-cells are permissive for this virus.

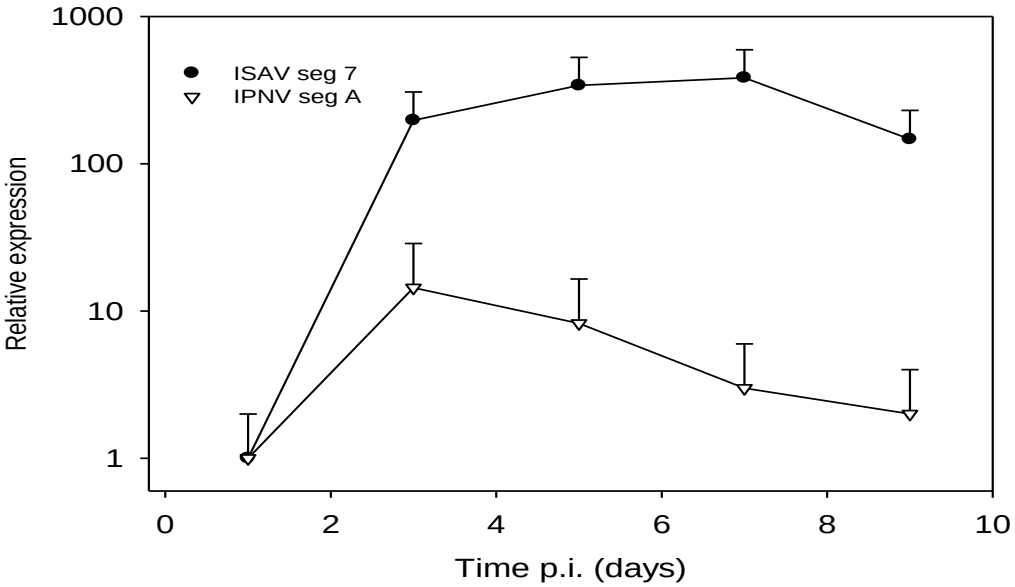

Figure 19: Relative mRNA transcription of IPNV segment A at 1, 3, 5, 7, and 9 days post infection.

As seen in Figure 1919 above, the transcription of IPNV segment A increased significantly until day 3 post infection relative to day 1 post infection, with a factor of 14.38, p-value: 0.021. However, at later sampling points, the mRNA levels for segment A was reduced to levels observed at day 1 post infection, probably reflecting the strong cytopathic effect of this virus on ASK-cells (see Figure 17).

4.3: Western blotting

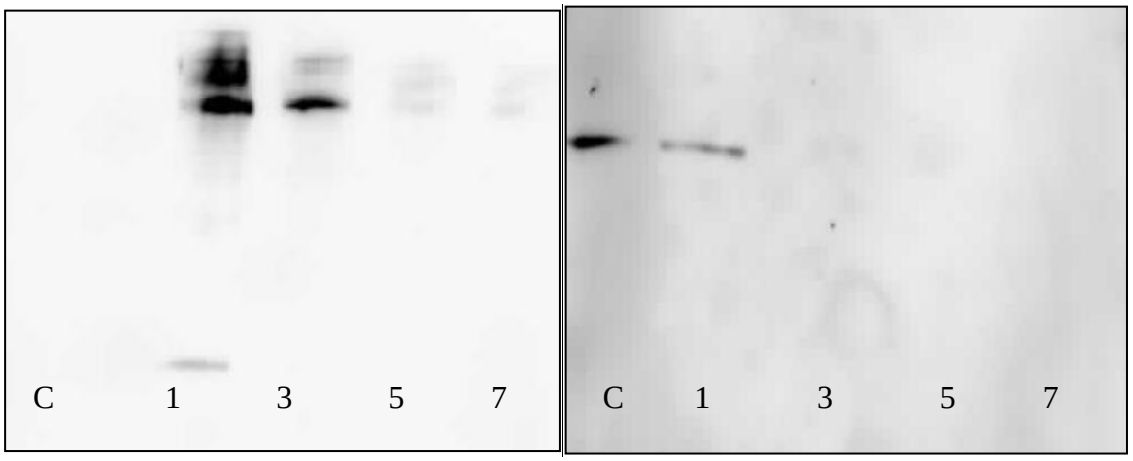

Figure 20: Left panel: Western blot of IPNV VP3. Included are control, and days 1, 3, 5, and 7 post infection. **Right panel:** Western blot with the same samples with antibodies against actin.

From the western blots seen above, the control with no virus shows no bands for VP3. From the western blot it is also apparent that VP3 is synthesized in the IPNV infected cells already from day 1 post infection indicating that this is a viral protein that is synthesized early after infection. The following days the bands appear weaker that could suggest that the transcription of this protein is reduced after the first day, but the most likely explanation is that as the viral infection proceeded and CPE were more extensive, the samples were harvested at a less efficient level than for the first days. For the western blot where antibodies against actin were used, the control shows a clear band, which can also be seen for day 1 post infection. For days 3, 5, and 7 post infection no bands can be readily observed, which may account for loss of harvested cells due the extensive CPE observed in the samples. When harvesting the samples, the media was removed. This media contained a significant amount of detached cytopathic cells, and was for this reason centrifuged, and the cell debris pellet was lysed together with the cells still attached to the wells. This was done to extract as much of the samples as possible, but as the western blots show, this was obviously not done efficiently enough.

Despite several attempts at western blots with ISAV antibody, no successful blot could be produced to confirm ISAV replication at the protein level.

4.4: Relative expression of target genes in Poly I:C-stimulated cells

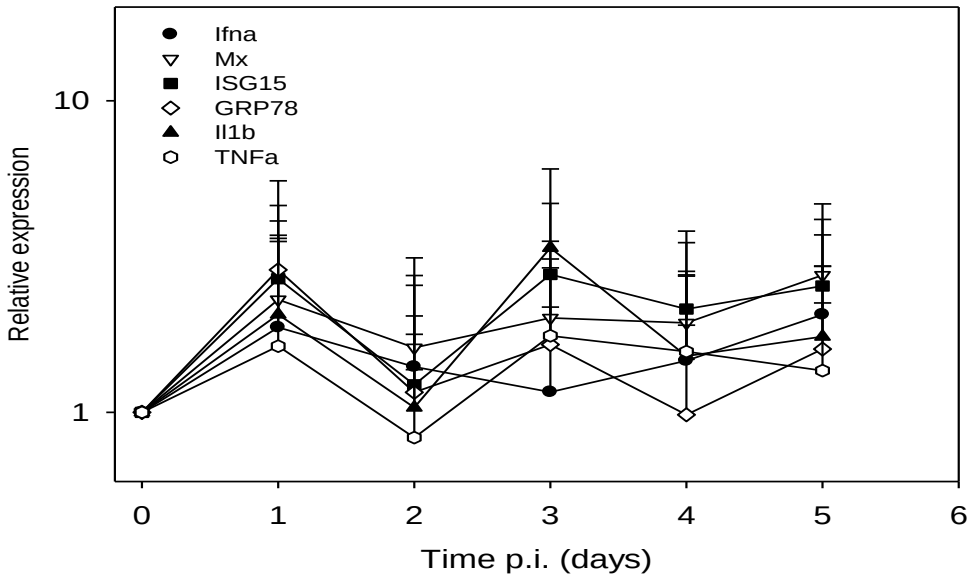

Figure 21: Effect of poly I:C stimulation (10 ug/ml) on relative expression of target genes. Each point represents 4 measurements from 2 independent experiments and is calculated relative to 2 reference genes (18S and EF1α) using ΔΔCt method.

To verify that the ASK cells were responsive to an established inducer of the interferon pathway we treated our cells with 10 ug/ml doublestranded polyI:C RNA and analysed transcription of a selection of inflammatory and interferon induced genes (ISGs). Despite a rather "bumpy" appearance of the time series data, the general trend was that poly I:C treatment of the ASK cells induced significant overexpression of the investigated transcripts at most time points (Figure 21). The relative expression of IFNa was significantly up-regulated on day 1 and day 5 after poly I:C stimulation, displaying a bimodal curve with peaks on these days. However on day 2 and day 3 post stimulation, the relative expression of IFNa decreases slightly before finally increasing again at day 4 post stimulation. Also displaying this bimodal curve in relative expression is the target gene Mx. Mx was significantly up-regulated on day 1 post stimulation (p-value: 0.033) with an expression ratio of 2.3, and on day 5 post stimulation with an expression ratio of 2.76 (p-value: 0.001), showing that IFNa and Mx expression was induced early by the poly I:C stimulation before it decreases and then being up-regulated at a later time point. ISG15 was induced from day 1, but the increase in expression was not significant until day 3 post stimulation with a relative expression ratio of 2.77 (p-value: 0.001). The relative expression of GRP78 was significantly up-regulated at day 1 post stimulation, but then decreased to control levels . Another gene that was significantly up-regulated was TNFa on day 3 post stimulation with poly I:C, with an expression ratio of 3.37 (p-value: 0.023). The relative gene expression of IL-1β did not significantly change during these five days post stimulation with poly:IC.

4.5: Effect of viral infection on transcription of genes involved in inflammation

The relative expression of the six virus responsive genes presented here has been normalized against two reference genes; elongation factor 1 alpha, and 18S by using the ΔΔCq-method. The relative expression levels have been set to one for day 0 (control), as this is the base line level.

4.5.1: Relative expression of target genes in ISAV-infected cells

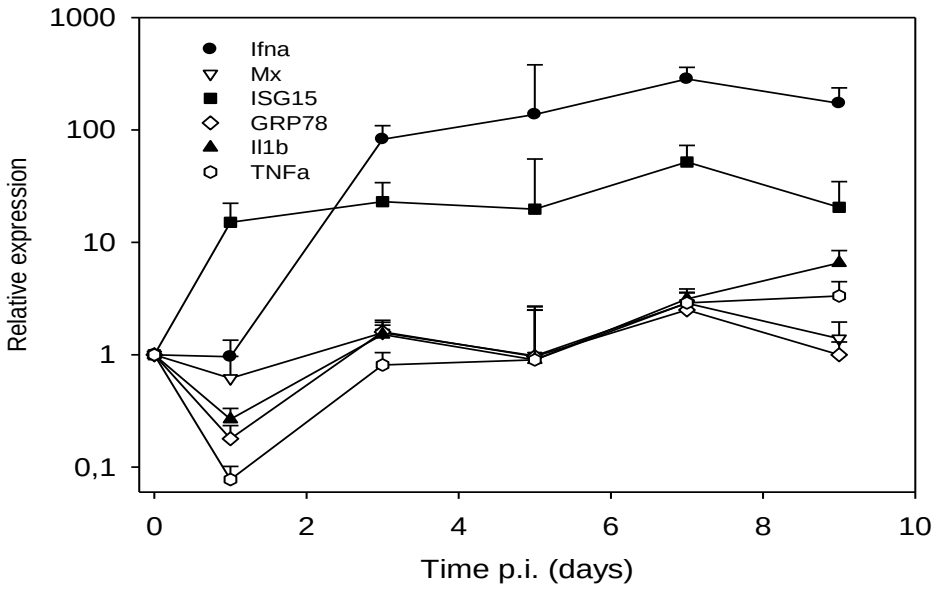

Figure 22: Effect of ISAV infection in ASK-cells on relative expression of inflammatory transcripts analyzed by Q-PCR. Each point represents 8 measurements from 2 independent experiments and is calculated relative to 2 reference genes (18S and EF1α) using ΔΔCt method.

At day 1 post infection with ISAV an increase in the relative expression of the ISG15 target gene was observed (Figure 22). A distinctive decrease in the relative expression levels for GRP78, IL1β and TNFa was apparent 1 dpi (p-value: 0.020, 0.026, 0.028 respectively) but these levels increased significantly on day 7 post infection for all three genes (p-value: 0.003, 0.002, 0.011 respectively). IFNa was significantly up-regulated from day 3 post infection (p-value 0,001) and steadily increased until a peak was reached at day 7 post infection with a relative expression ratio of approximately 284,5 (p-value 0,007). ISG15 was the only target gene that was significantly up-regulated at day 1 post infection with an expression ratio of 15.1 (p-value: 0.001). Its expression further increased on day 3, and reached a peak on dpi 7, with an expression ratio of 51.8 (p-value: 0.001). Its expression was significantly up-regulated throughout the nine days post infection. ISG15 apparently follows a similar expression pattern to IFNa, but is induced earlier than IFNa by the ISAV-infection, which may suggest that there exists alternative mechanisms of inducing ISG15 than IFNa-induction alone. The TNFa gene was down-regulated on day 1 post infection (expression ratio: 0.078, p-value: 0.014), but not significantly different from control later in infection. The pro-inflammatory cytokine IL-1β was significantly down-regulated on day 1 post infection with an expression ratio of 0.27 (p-value: 0.026), significantly up-regulated on dpi 3 with the expression ratio 1.52 (p-value:

0.001) and unchanged at 5 dpi, before a significant increase on dpi 7 and 9. IL-1β showed the highest up-regulation on day 9 post infection with an expression ratio of 6.57 (p-value: 0.001) showing that an inflammatory reaction in the ASK-cells is in fact induced in these ISAV-infected cells.

4.5.2: Relative expression of target genes in IPNV-infected cells

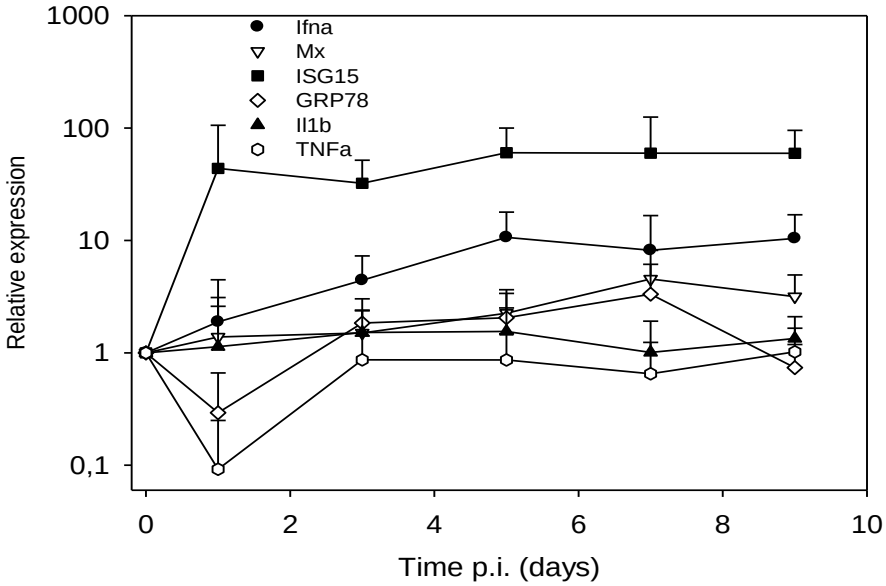

Figure 23: Effect of IPNV infection in ASK-cells on relative expression of inflammatory transcripts analyzed by Q-PCR. Each point represents 8 measurements from 2 independent experiments and is calculated relative to 2 reference genes (18S and EF1α) using ΔΔCt method.

As with the ISAV-infected ASK-cells, a strong down-regulation of the pro-inflammatory TNFa-gene was observed with an expression ratio of 0.092 (p-value: 0.001) on day 1 post infection with IPNV (Figure 23). There was also a significant down-regulation of the expression of the chaperone protein GRP78 on day 1 post infection (expression ratio: 0.293, p-value: 0.001). A relatively strong up-regulation of the ISG15 gene throughout the infection was observed with IPN-virus. ISG15 was significantly up-regulated from day 1 post infection with an expression ratio of 43.8 (p-value: 0.130), reaching an expression ratio of 60.3 (p-value: 0.012) on day 5 post infection. It remained nearly unchanged until day 9. Interestingly the relative expression of the IFNa gene was not as strongly up-regulated by IPNV-infection as observed with ISAV-infection. The IFNa gene was significantly up-regulated from day 3 post infection with an expression ratio of 4.43 (p-value: 0.028), reaching the highest expression ratio of 10.66 (p-value: 0.017) on day 5 post infection. The relative expression of the antiviral Mx protein increased steadily from day 1 post infection, and reached a peak at

day 7 with an expression ratio of 4.54 (p-value: 0.001). It was also observed that the chaperone protein GRP78 was significantly up-regulated from day 5, reaching a peak on day 7 post infection with an expression ratio of 3.33 (p-value: 0.001) before decreasing again at day 9.

4.6: Effect of transfection on transcription of genes involved in inflammation

4.6.1: Relative expression of target genes in RIG-I-transfected cells

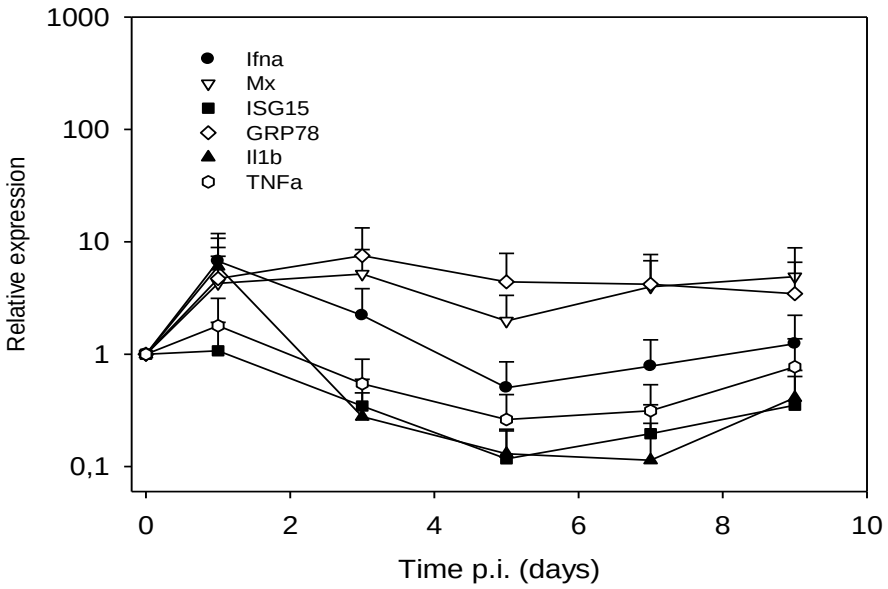

Figure 24: Effect of RIG-I transfection in ASK-cells on relative expression of inflammatory transcripts analyzed by Q-PCR. Each point represents 8 measurements from 2 independent experiments and is calculated relative to 2 reference genes (18S and EF1α) using ΔΔCt method.

On day 1 post transfection with the RIG-I plasmids (using the electroporation transfection method) a significant increase in the relative expression of IFNa was observed with IFNa reaching an expression ratio of 6.72 (p-value: 0.007) (Figure 24). At later time points, the IFNa expression was not different from control. The expression of the Mx-gene was significantly up-regulated between 4,27 fold and 4,90 fold all sampling points after RIG-I transfection. The Mx expression pattern followed that of the IFNa expression, but its up-regulation was sustained during the sampling period. Interestingly the ISG15 gene expression was not induced despite of the IFNa being up-regulated day 1 post transfection.

The chaperone protein GRP78 was significantly up-regulated following transfection with the RIG-I-plasmid from day 1 till day 9.

The pro-inflammatory cytokine IL-1β was significantly up-regulated day 1 post transfection (expression ratio: 6.04, p-value: 0.001) before a strong and significant down-regulation was observed at days 3, 5 and 7 post infection. The up-regulation observed at day 1 post transfection is not unexpected, as the electroporation method in itself is expected to induce a transient inflammation. The pro-inflammatory cytokine TNFa is also significantly down-regulated on day 5 (expression ratio: 0.26, p-value: 0.013) and day 7 (expression ratio: 0.31, p-value: 0.011) post transfection.

4.6.2: Relative expression of target genes in MAVS-transfected cells

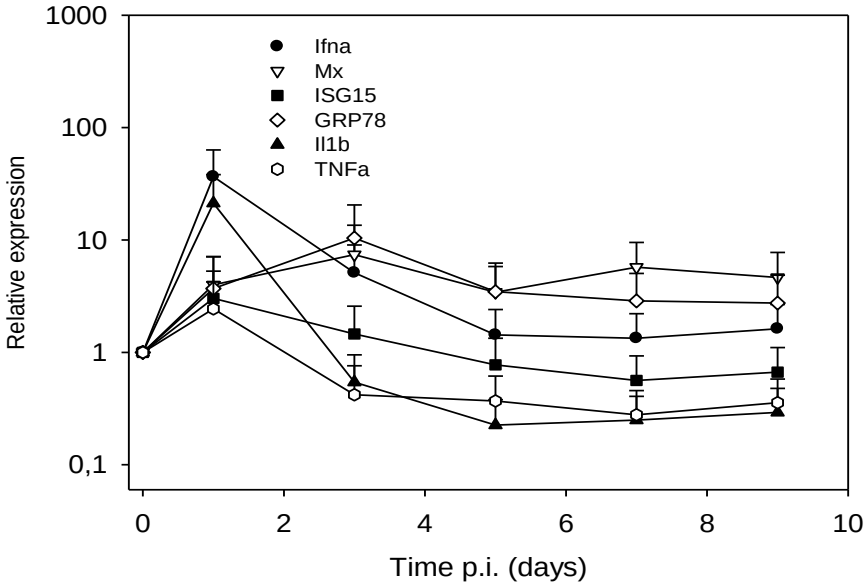

Figure 25: Effect of MAVS-transfection in ASK-cells on relative expression of inflammatory transcripts analyzed by Q-PCR. Each point represents 8 measurements from 2 independent experiments and is calculated relative to 2 reference genes (18S and EF1α) using ΔΔCt method.

On day 1 post transfection a strong up-regulation of IFNa was observed with an expression ratio of 36.53 (p-value: 0.001) (Figure 25). After 1 day post transfection the relative expression levels of this gene decreased rapidly to a ratio of 5.11 (p-value: 0.001) at day 3 post transfection and then further decreases.

The antiviral Mx gene was significantly up-regulated from day 3 post transfection. The ISG15 gene did not significantly change during the 9 days after transfection, but its expression seem to correlate with that of the IFNa gene.

GRP78 was significantly up-regulated by MAVS overexpression during the sampling period.

The pro-inflammatory cytokine IL-1β was significantly up-regulated on day 1 post transfection, with an expression ratio of 21.23 (p-value: 0.001). However the expression ratio fell to control values at later time points.

4.7: Comparison of the different target gene expressions as a result of infection and transfection:

To better visualize the effect of the different treatment on interferon related transcripts figure 26 shows the same data (Figure 22-25) as above for IFNa in aggregated plot.

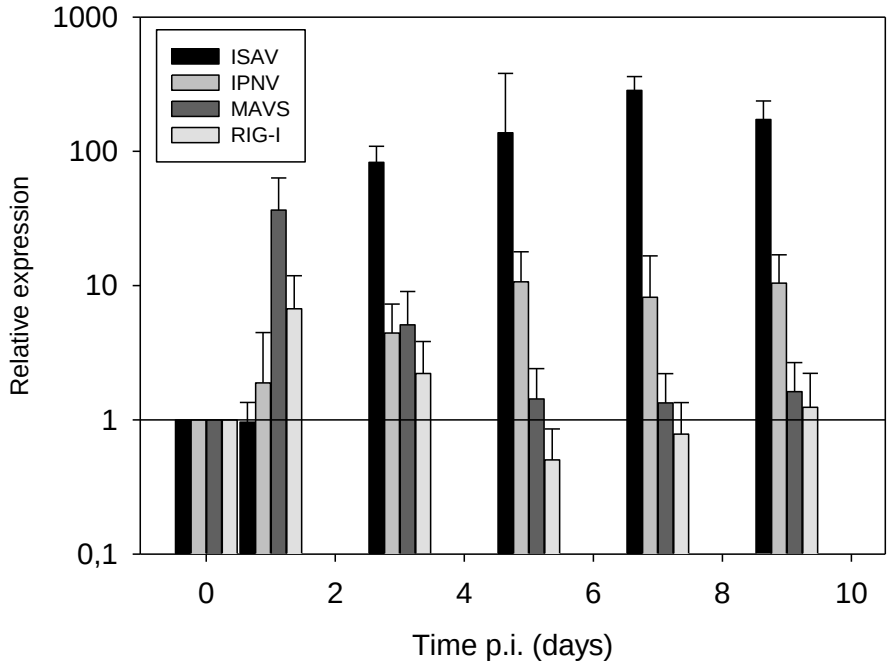

Figure 26: Comparison of the relative IFNa gene expression following different treatments (data from figure 22-25).

When the temporal pattern of IFNa expression after infection or RIG-I/MAVS overexpression was compared it can be seen that overexpression resulted in a more rapid, but transient IFNa induction compared to viral infection (Figure 26)**).** At day 1 post treatment the highest induction of the IFNa gene can be seen with the MAVS-transfection, with the RIG-transfection on second place. The ISAV-infection did not significantly induce the IFNa gene on day 1 post infection. Relatively low expression ratios of IFNa can be observed with the IPNV-infection compared to the ISAV-infection. ISAV-infection clearly induced a strong IFNa up-regulation compared to both transfections and the IPNV-infection. The lowest IFNa

induction can be associated with RIG-I-transfection, and even a slight down-regulation can be observed at day 5 post transfection.

The same was true for the kinetics of MX expression, but this transcript was sustained at an elevated level after all treatments (Figure 27). However, the other interferon regulated transcript, ISG15, was mainly stimulated by viral infection and not by RIG-I overexpression (Figure 288). The chaperone GRP78 was much more stimulated by protein overexpression than by viral infection throughout the observation period (Figure 29). Transcription kinetics for the cytokines IL-1β and TNFa was also preferentially induced by MAVS/RIG-I overexpression with a strong burst at day 1 followed by levels at, or below control at the later stages (not shown).

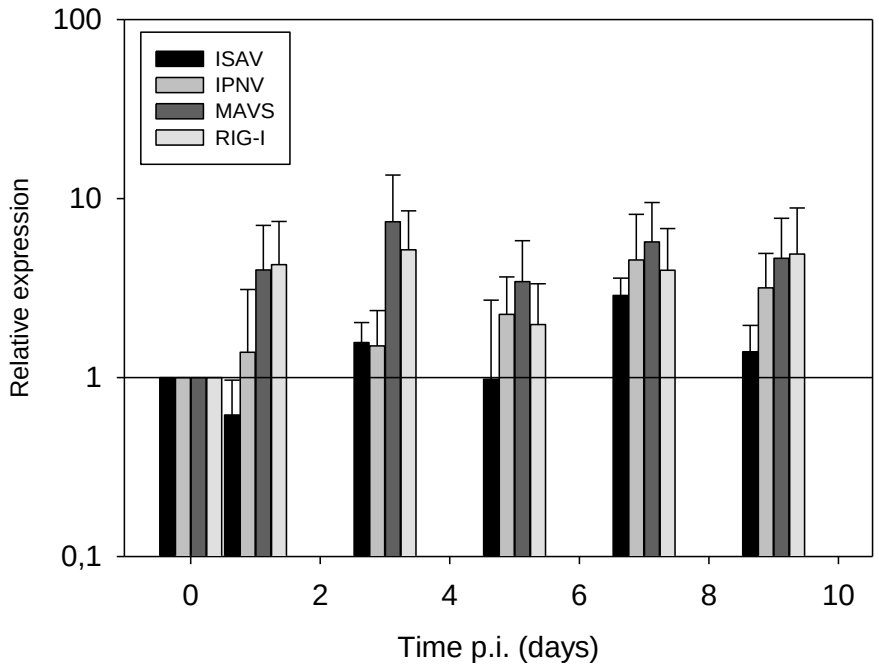

Figure 27: Comparison of the Mx gene expression following different treatments (data from figure 22-25).

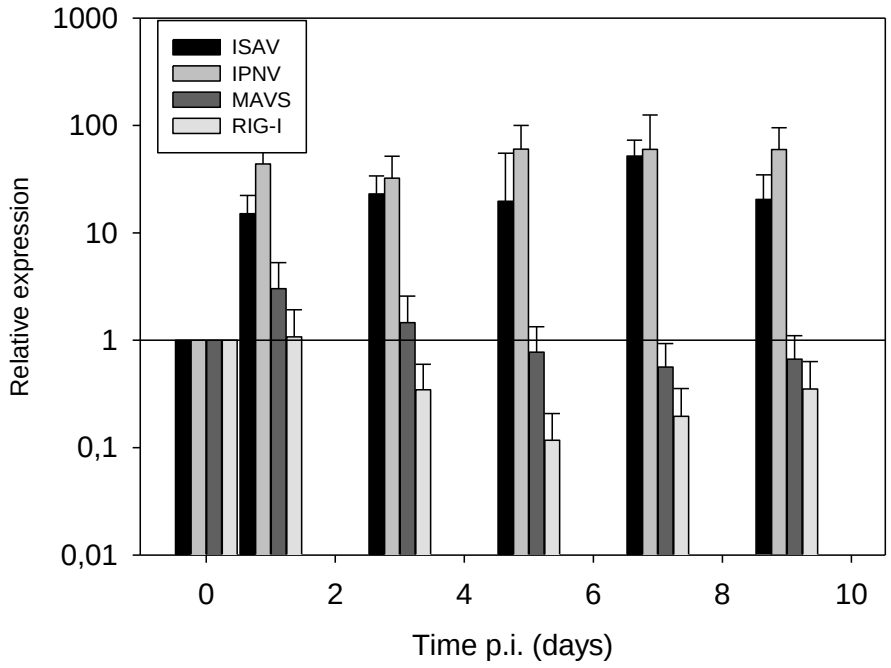

Figure 28: Comparison of the ISG15 gene expression following different treatments (data from figure 22-25).

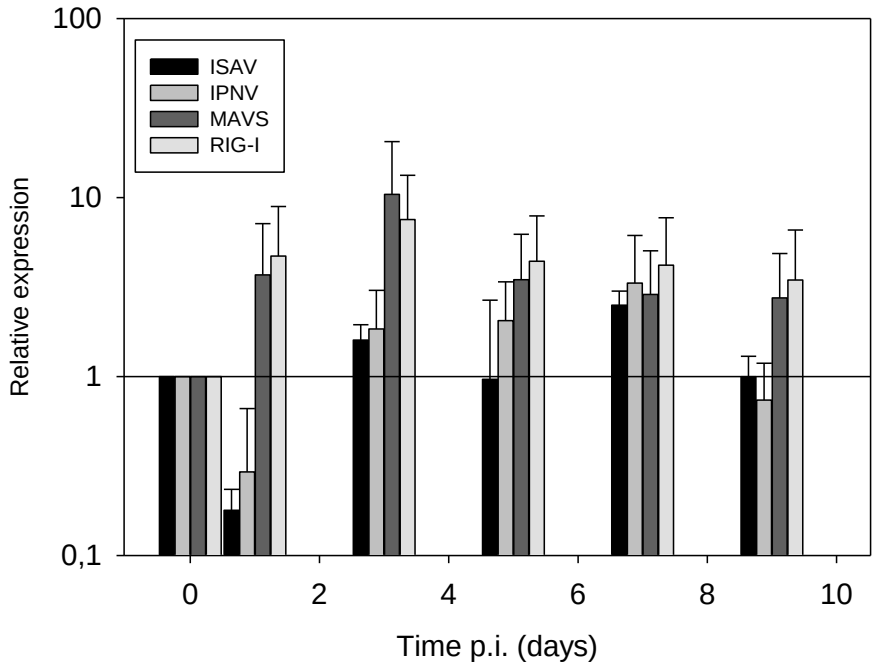

Figure 29: Comparison of the GRP78 gene expression following different treatments (data from figure 22-25).

4.8: Transfection of cells

Although the intended purpose of plasmid transfection with RIG-I and MAVS genes was to increase their protein expression levels in the cells, the transfection procedure and the introduction of plasmid DNA into cytoplasm may have other effects on the cells. Several times during the work with this thesis, it was observed that transfection with the full-length RIG-I plasmid caused significant cell death compared to transfections done with the MAVS-plasmid although both these inserts were in the same expression vector (pcDNA1.1/Amp) (Figure 30). The transfections were done with the same plasmid concentrations under the same conditions using the same amount of cells, and this was a consistent observation. The electroporation as a transfection method is associated with a significant loss of cells, but fewer viable cells were observed when transfecting with pcDNA1.1/Amp with the RIG-I-insert. One possible explanation could be the size of the insert in this pcDNA1.1/Amp-vector as the MAVS-insert is almost 2 kb, whereas the RIG-I-insert is nearly 4 kb long.

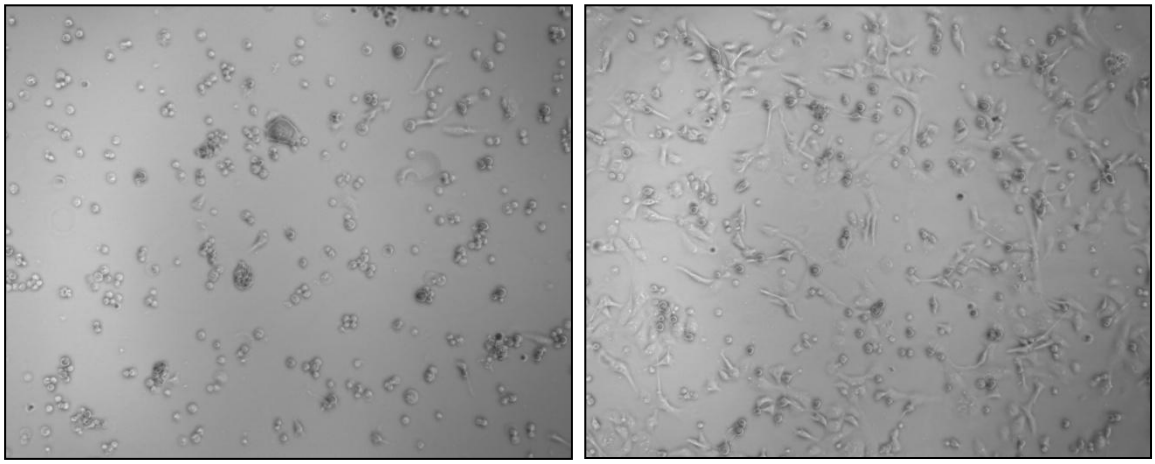

Figure 30: Left: ASK-cells transfected with pcDNA1.1/Amp-RIG-I. **Right**: ASK-cells transfected with pcDNA1.1/Amp-MAVS. 10X magnification. 24 hours post transfection with 1µg of plasmid-DNA

4.8: Fluorescence microscopy

4.8.1: The subcellular localization of RIG-I-GFP and MAVS-GFP

CHSE-214 cells were transfected with plasmids containing RIG-I-GFP and MAVS-GFP inserts to study the distribution and subcellular localization of these proteins in the cell.

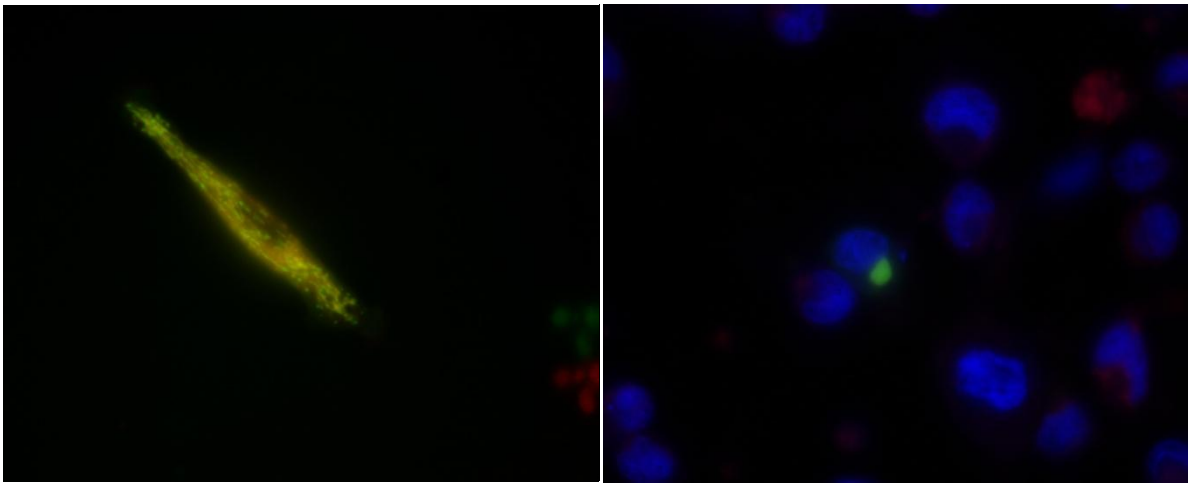

Figure 31: Fluorescence micrographs of CHSE cells expressing MAVS-GFP. Right panel**:** MAVS-GFP (green color) together with MitoTracker Red (red color) and DAPI (blue color) . **Left:** MAVS-GFP and MitoTracker Red overlay showing colocalization (yellow).

In cells transfected with MAVS-GFP the green label appeared to co-localize with the MitoTracker in many of the transfected cells (as MAVS is a mitochondrial membrane protein) like in Figure 31, left panel. The co-localization of GFP and MitoTracker red produced a yellow color when studying the mounts in the fluorescence microscope. However, it was frequently observed that when overexpressing MAVS-GFP, this protein could be observed as blebs near the nucleus of the cell, as seen in the left panel of figure 32. This was often observed from 2 days post transfection with MAVS-GFP. This could indicate that when the cells were overexpressing MAVS, the transport system where newly synthesized proteins are transported out from the ER is saturated and this protein is not evenly distributed as it would be at normal expression levels. There was no co-localization of MAVS-GFP with the lysosomal marker (figure 32, right panel).

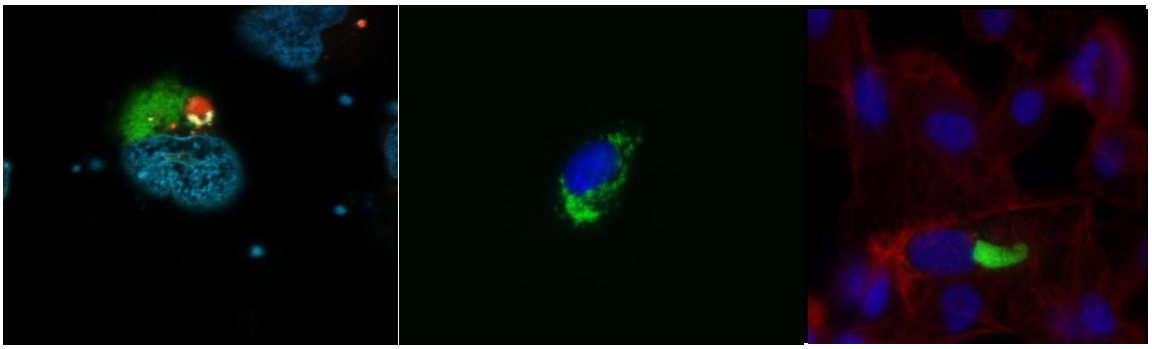

Figure 32: Right: Fluorescence micrographs of CHSE cells expressing MAVS-GFP. Right panel: MAVS-GFP (green), DAPI (blue) and LysoTracker (red) Middle panel**:** MAVS-GFP (green) and DAPI (blue) Left panel**:** MAVS-GFP (green), phalloidin (red), DAPI (blue)

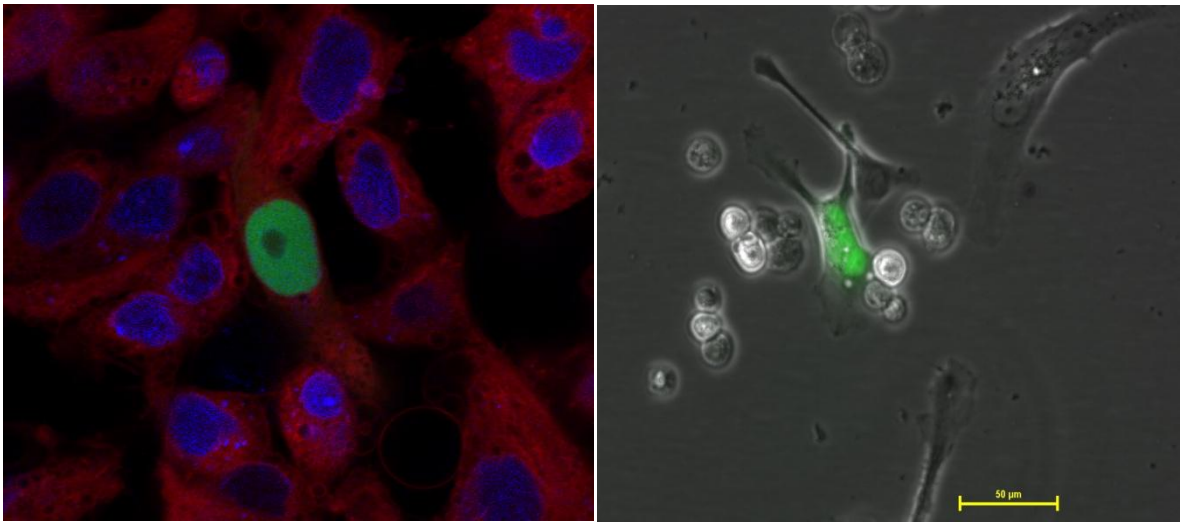

Figure 33: Fluorescence micrographs of CHSE cells expressing RIG-I-GFP. Right panel**:** Phase contrast of RIG-GFP transfected CHSE cells. Left panel : RIG-I (green), DAPI (blue) and Cellmask (red).

When transfecting with RIG-I-GFP, this protein showed an even distribution throughout the cytosol of the cell (figure 33 right panel). However more often than not, the RIG-I-GFP a couple of days after transfection, could be observed as vacuoles in close proximity to the cell nucleus. Sometimes RIG-I-GFP even showed a co-localization with the nucleus (figure 33 left panel). Overexpressing RIG-I may alter the natural distribution of this protein in these cells. Whether this is attributed to the fact that the proteins are overexpressed or that the GFP-tag attached to the RIG-I and MAVS may alter the function and distribution of them, remains unclear. One should also keep in mind that the RIG-I-GFP is not the full-length RIG-I but the N-terminal end fused with the GFP and that this may also contribute to a possible alteration of subcellular distribution.

4.8.2: Effects of transfection on IPNV and ISAV infection

CHSE-cells were transfected with RIG-I-GFP and MAVS-GFP and subsequently infected with IPNV and ISAV (MOI of 1) to investigate if the transfection could have any positive effects in offering the cells any protection against infection with these two pathogenic viruses.

First, the degree of infection was analysed by quantitation of immunopositive cells in the microscope at 24 h after inoculation. The infection degree was by staining the nuclei of cells with DAPI to count the total amount of cells, compared to infected cells (stained with fluorescent antibodies against the viruses).

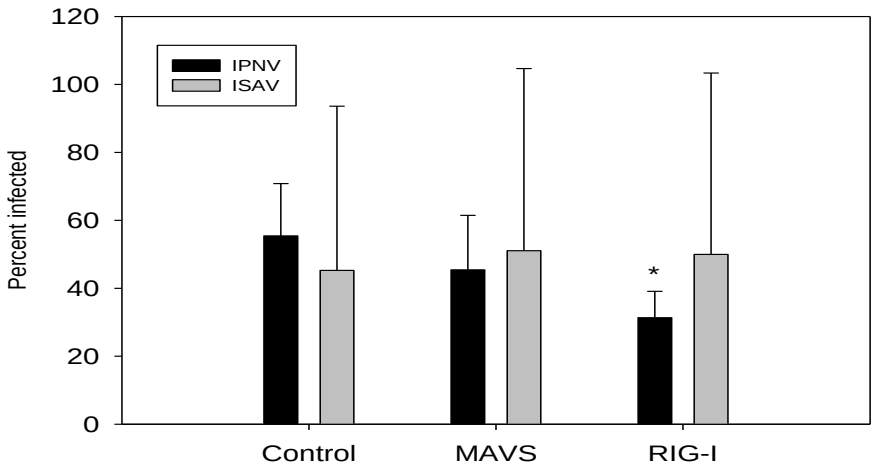

Figure 34: Percent infected cells (immuno fluorescence positive cells) at 24 h p.i. in CHSE control cells and in cells overexpressing MAVS or RIG-I. Bars show mean % immunopositive cells ± SD (n= between 200 and 600 in each group) (star denotes value significantly different from control $p<0.05$)

Although it may seem like RIG-transfection conferred some protection against ISAV infection, this turned out to be difficult to determine as for all the transfected samples. The same quantitations were performed for later time points p.i., but the infected cells were washed out by the washing steps involved in immuno-staining. The reason for this was probably that the cells did no longer adhere strongly to the chamber after 3-5 days of infection, and transfection seemed to aggravate this effect. The loss of cells during staining procedures was too big to give any conclusive results for day 3 and 5, so only the results for day day 1 is commented and represented in Figure 34. Coating of the chambers with poly-L-

lysine was performed prior to splitting the cells into the chambers, and in addition to this the washing steps were shortened in an attempt to reduce the number of cells lost. This unfortunately did not reduce the number of cells lost due to staining procedures. In addition to this, the transfection degree obtained was often low, even after transfection with the electroporation method. One must assume that transfecting only a few cells will not protect the entire cell population. It was also observed that cells that were transfected (GFP positive) did get infected with both IPNV and ISAV.

Counting results were analyzed in SigmaPlot with ANOVA (analysis of variance). The differences in the median values among the treatment groups for ISAV samples were not great enough to exclude the possibility that the difference was due to random sampling variability; there was not a statistically significant difference between the groups (P = 0,514) i.e. the interpretation of this result was complicated by the ISAV samples not following a normal distribution.

However, the differences in the mean values among the treatment groups for IPNV samples were greater than would be expected by chance; and there was a statistically significant difference (P = 0,025) for control versus RIG-I as seen in Figure 34. There was no significant difference for control versus MAVS, and MAVS versus RIG-I.

4.9: $TCID_{50}$ titration

To analyse the effect of transfections on the secretion of virus to the cell culture supernatant (= virus production) the $TCID_{50}$ for the supernatant from the transfected and non-transfected cells infected with IPNV were determined. On day 3, medium from nontransfected cells infected with IPNV had a $TCID_{50}$/ml of $4.49x10^2$. After 5 days the levels of virus was in the same range ($TCID_{50}$/ml = $3.29x10^2$). For the MAVS transfected cells + IPNV on day 3 and 5 no $TCID_{50}$ could be determined as not enough wells were positive i.e. CPE had not appeared in sufficient number of wells. This also applies to the ISAV titrations were all wells were negative even after 5 weeks with incubation also for the non-transfected control samples. Below the titration-plates for IPNV are presented. The titration-plates for ISAV are not presented as they are all negative and could not be used.

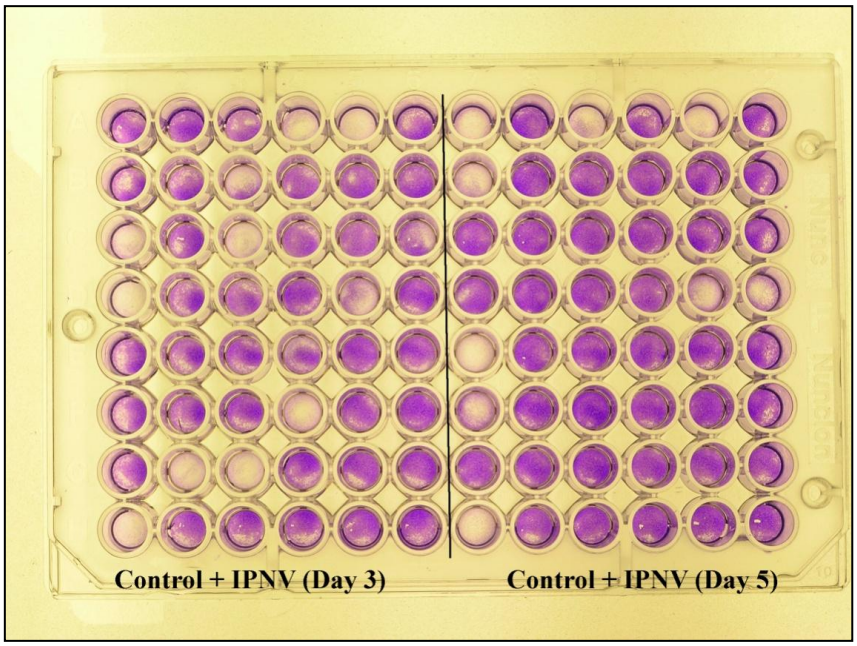

Figure 35: The 96-well plate used for $TCID_{50}$ titration of the control samples infected with IPNV.

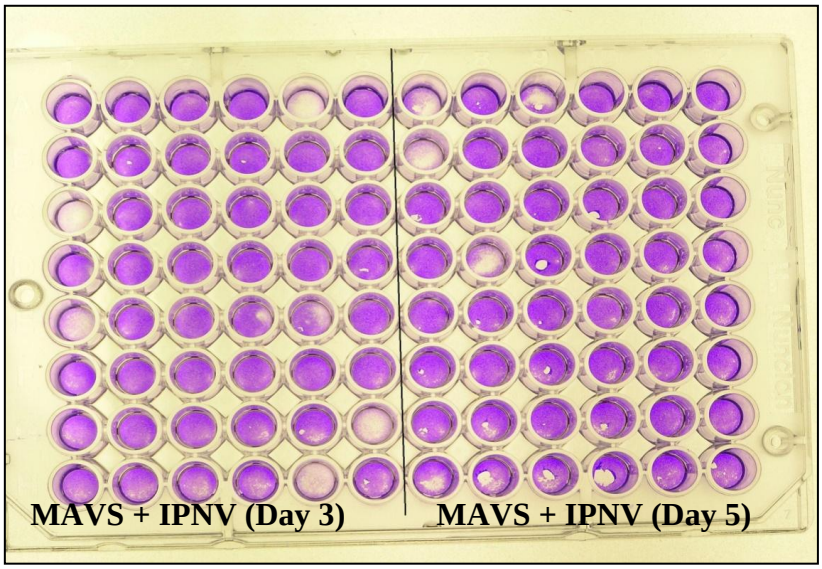

Figure 36: The 96-well plate used for $TCID_{50}$ titration of MAVS transfected cells infected with IPNV.

As an alternative to the failed $TCID_{50}$ calculation, the relative number of intact cells was determined by spectrophotometric analysis of the cellular stain. After the plates were photographed under a light box, the crystal violet dye was redissolved in 100 µl 1% SDS solution and the optical density (OD) was read at 590 nm with the HTS 7000 Plus, Bio Assay Reader from Perkin Elmer. The optical densities at 590 nm were plotted in excel® and the

means and standard deviations of 8 wells per virus dilution , (SD) and standard error (SE) were calculated before the graphs were made. In the graphs below, the data shown are average optical densities ± SE against the log10 dilutions.

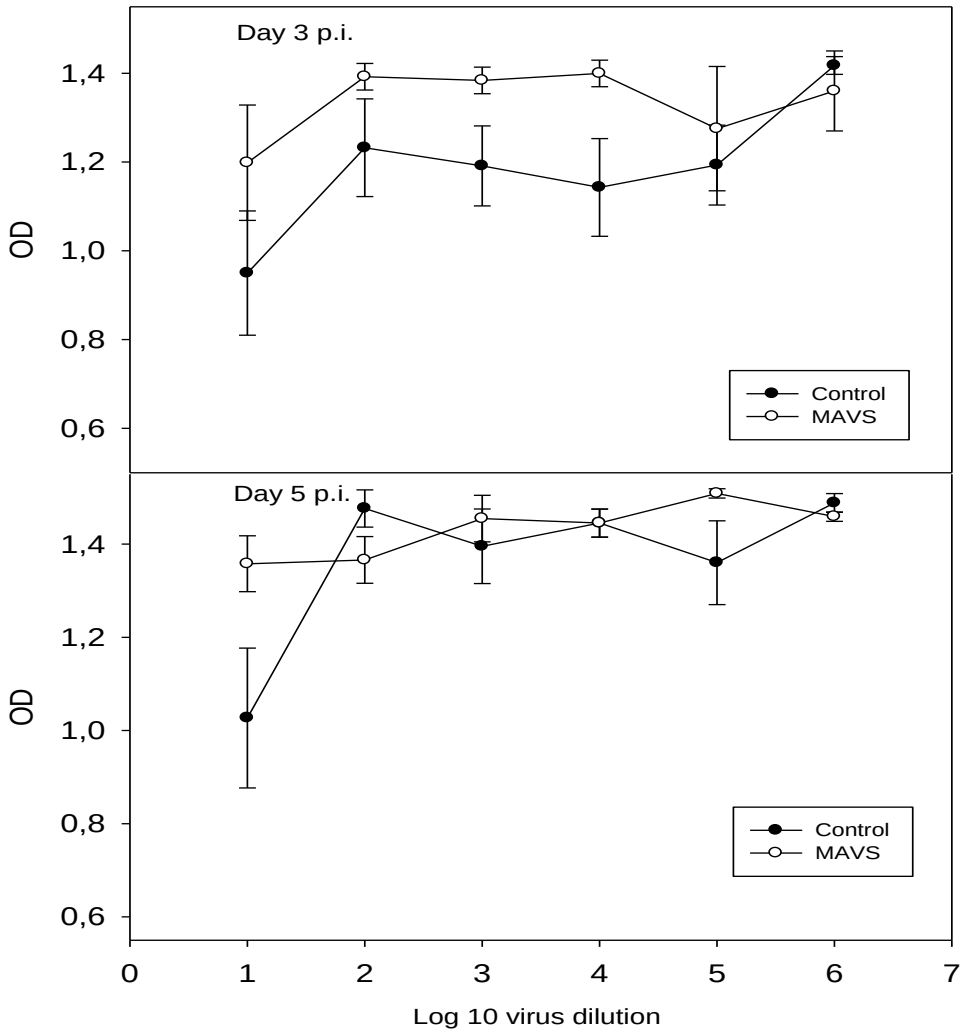

Figure 37: Average optical densities ± SE for the viral titration plates.

As the graphs above show, the average optical densities increase with increasing dilution factor. This could be expected as more dilute virus supernatants should produce a less prominent CPE than less dilute virus supernatant and leave more viable cells available for the crystal violet staining. Although no $TCID_{50}$ could be determined for the samples with MAVS transfected cells infected with IPNV, transfected cells have a generally higher OD (more viable cells?) than the control samples infected with the same virus MOI. However unfortunate it is that the $TCID_{50}$ could not be determined for the MAVS samples, this viral

titration experiment may suggest that MAVS-transfection and overexpression in the cells may confer *some* level of protection against IPNV. But further investigations are needed to confirm this hypothesis.

As mentioned earlier, no $TCID_{50}$ could be determined for ISAV4. Although the CHSE-cells has in previous studies been confirmed to be permissive for ISAV [50], and this particular ISAV strain has been demonstrated to be associated with relatively high mortality [10], it did not produce a significant CPE in the $TCID_{50}$ titration, even after 7 and 8 weeks. This could be due to the number of passages the CHSE-cells had, as it has been shown that after the cells has been split many times, they become refractory to infection with ISAV. However changing to a new CHSE-cells with a lower passage number, did not solve the problem. The number of times the virus has been passaged is also of great importance. Every time the virus replicates, non-infective or defective virions are assembled, and after a sufficient number of passages, the virus inoculate may no longer be as infective or pathogenic as the first isolate. This could be a possible explanation to why no prominent CPE was observed with this virus during the work with this thesis.

5: Discussion

The innate immune system is the first line of defense against infectious pathogens and is especially critical for the teleost fish as the adaptive immune response is not as specific as the mammalian system. Surveillance mechanisms involve pattern recognition receptors (PRRs) on the cell surface and in the cytoplasm. Three families of PRRs have been identified to date; the TLRs, the NLRs and the RLRs. The RLR signaling pathway induces an antiviral immune response upon recognition of viruses, including the production of pro-inflammatory cytokines and type I IFNs.

In this thesis salmon cell lines were infected with ISAV and IPNV to study the intracellular biochemical changes that is initiated during an infection with these aquatic pathogenic viruses. The importance of RIG-I and MAVS on virus replication and the development of CPE in the cell lines were investigated by transfecting cells with plasmids containing these inserts. The GFP-variants of these proteins were used to transfect cells to examine the subcellular localization of these proteins in the cell lines ASK and CHSE-214.

5.1: ASK- and CHSE-cells

Both ASK- and CHSE-cells were shown to be permissive cell lines for the IPN- and ISA-virus replication for the strains that were used, producing an effect in infected cells as seen in Figure 17 and Figure 18. The western blot of IPNV also verifies that IPNV does replicate in CHSE-cells **(**Figure 20**)** which has been confirmed previously in several studies [66, 67]. The Q-PCR results of ISAV segment 7 and IPNV segment A (Figure 19**)**, also confirms that there indeed is a replication of these viruses in ASK-cells. ISAV has previously been confirmed to replicate in CHSE-cells [9, 17, 68] and ASK-cells [30] which was also confirmed during the work with this thesis.

Furthermore it was investigated whether the ASK-cells were responsive to an established inducer of the interferon pathway, by treating our cells with 10 ug/ml double stranded poly I:C RNA and analyzing transcription of a selection of inflammatory and interferon induced genes (ISGs). This was included as a positive control to confirm that the ASK-cells contained all the components of the signaling pathway needed (see Figure 21**)**.

5.2: ISAV infection

The ISAV4 strain used in this thesis did not produce any prominent CPE in ASK-cells or CHSE-cells that were used, although this ISAV strain has been demonstrated to be associated with high mortality in salmon [10]. This could be due to high passage number of the cell lines used, as this could cause the cells to be refractory to ISAV infection, but this is probably not the explanation as changing the cells to a new batch with lower passage number did not aid in any way. A more probable explanation is that the virus may have been attenuated when it was propagated in ASK-cells, as every replication cycle for the virus, carries with it the risk of defective and non-infective virions to be produced and assembled. The virus supernatant used in the infection trials in this thesis, were from a virus supernatant propagated in ASK-cells where they produced a cytopathic effect after 10 days. However this new virus supernatant harvested from this virus propagation did not produce any further CPE when inoculated in new infection experiments. Although neither the ASK- or the CHSE-cells died when inoculated with ISAV4 during this work, the cells did however change their appearance, which could be readily observed in a phase contrast microscope. The cells did not detach or round up, but the ISAV4-infection did induce the cells to become vacuolarized, and induced a granular appearance (especially pronounced at day 5 post infection) as shown in Figure 18.

Our *in vitro* studies showed that ISAV is an early and powerful inducer of interferon and interferon induced genes Mx and ISG15. This is consistent with the findings of Kileng *et al.* and Jensen *et al.*[69, 70] in earlier studies. ISAV-infection induced a significant IFNa up-regulation in ASK-cells. IFNa was significantly up-regulated from day 3 post infection (p-value 0,001) and steadily increased reaching a peak at day 7 post infection with a relative expression ratio of approximately 284,5 (p-value 0,007). Although the IFNa gene was up-regulated by the ISAV infection as seen in Figure 22, it has been suggested in previous studies that even though this gene is up-regulated by the infection with this virus, it does not protect against the ISAV infection [30], in fact interferon-signaling antagonist viral proteins have been described [71, 72]. These proteins could be used by the virus as a strategy to evade the IFN system as has been described for mammalian viruses [73]. These results appear to indicate that induction of type I IFN and IFN dependent genes in ISAV infected cells may not provide protection against the virus.

IL-1β was significantly up-regulated at 7 and 9 dpi in ASK-cells. This has also been observed in a previous study performed *in vivo* in ISAV infected fish [74]. This may suggest that IL-1β can have a role during ISAV infection as it has been described for other orthomyxoviruses like Influenza A [75].

ISAV infection significantly up-regulated the transcription of ISG15 as seen in Figure 22. A study by Røkenes *et al.* [76] demonstrated the immunoprecipitation of ISAV protein from infected TO cells using anti-Atlantic salmon ISG15 anti-serum, which suggested that binding between the ISG15 and the ISAV nucleoprotein occurred. This suggests the possible antiviral role of Atlantic salmon ISG15 in ISAV infection of Atlantic salmon.

5.3: IPNV infection

IPNV readily produced a pronounced CPE in both ASK- and CHSE-cells when infecting the cells with a virus MOI of 1. All infection trials performed in this thesis were done with an MOI of 1. Although both the ISAV and IPNV were inoculated in cells with the same MOI, IPNV gave a more pronounced pathogenic effect on the cells compared to ISAV. Total CPE with IPNV was usually achieved after 3-4 days post infection, and the cells were clearly detached from the surface being more refractile (brighter) under phase contrast microscopy as seen in Figure 17. Interestingly CHSE-cells infected with IPNV showed a morphological change before the CPE was evident, in form of disruption of the cell membrane. This has also been observed in a previous study [67], where the authors suggested that the disruption observed of the cell membrane may be attributed to apoptosis of the cells. Host cell apoptosis is part of the innate immune response to virus infection, serving to limit the progeny production of the virus [67].

A significant up-regulation of GRP78 was observed during IPNV infection of ASK-cells. This may suggest that the lumen of the endoplasmatic reticulum of the cell experiences an increase in newly synthesized proteins (perhaps viral proteins) being translocated to the ER, or it may be an up-regulation of GRP78 induced to protect the cell against apoptosis (programmed cell death). This might be an intended effect of the IPN-virus, as apoptosis induced at the wrong time point may interfere with the virus replication. The relative expression of the pro-inflammatory cytokines IL-1β and TNFa remains relatively unchanged during all sampling points, suggesting that IPNV does not induce an inflammatory reaction in

ASK-cells. This is also consistent with the findings of another study, where IPNV infection in Atlantic salmon smolts failed to induce IL-1β and TNFa [30, 74].

Interestingly the relative expression of the IFNa gene was not as strongly up-regulated by IPNV-infection as observed with ISAV-infection. The IFNa gene was significantly up-regulated from day 3 post infection with an expression ratio of 4.43 (p-value: 0.028), and reached its highest expression ratio of 10.66 (p-value: 0.017) on day 5 post infection. It is clear that IPNV has evolved mechanisms to overcome the IFN responses. Viral proteins VP4 and VP5 seem the most probable candidates responsible for interfering with the IFN-signaling pathway in salmon [77]. Concerning interferon signaling, as McBeath *et al.*[74] indicated, the induction of the IFN system by IPNV seems to involve complex virus/host interactions and may play a role in determining states of resistance/susceptibility. Moreover, IFN signaling after IPNV infection may be dependent on the type of cell infected. However, IPNV may not have developed mechanisms to counteract IFN induced antiviral proteins because cells treated with Atlantic salmon IFN are effectively protected against IPNV induced lysis as demonstrated by Robertsen *et al.* [78].

ISG15 was significantly up-regulated during IPNV infection at all sampling points. This has also been observed in a previous study for both IPNV and ISAV [76]. Although ISG15 homologues have been identified in several fish species like crucian carp, goldfish, Atlantic salmon and Atlantic cod, little information exists about their biological properties [42, 76].

5.4: MAVS transfection

MAVS transfection in CHSE-cells appears to confer some protection against IPNV as demonstrated by higher OD-values from the $TCID_{50}$ titration but interpretation of these results were difficult due to few positive wells. However this effect was not observed when CHSE-cells were transfected with MAVS prior to immunostaining as presented in Figure 34 as the MAVS sample values did not significantly differ from the control values.

The target gene GRP78 was significantly up-regulated at all sampling points in MAVS transfected ASK-cells (Figure 29**)**. This was not unexpected, and suggests an induction of protein synthesis, which one could expect after a transfection with a plasmid that is transcribed inside the cell. The transfection itself influences the relative expression of target genes.

MAVS co-localized with the mitochondrial marker as shown in 31, confirming that it shares this essential location with its mammalian counterpart**.** This has also been demonstrated in several studies on MAVS [34, 37, 38]. However, it was frequently observed that when overexpressing MAVS-GFP, this protein could be observed as blebs near the nucleus of the cell, as seen in Figure 32. This was often observed from 2 days post transfection with MAVS-GFP. This could indicate that when the cells were overexpressing MAVS, the transport system where newly synthesized proteins are transported out from the ER is saturated and this protein is not evenly distributed as it would be at normal expression levels. Another explanation is that overexpression of MAVS-GFP by transfection, induces a stress response in CHSE-cells and forms large aggregates as discussed by Hou *et al. [37]*.

5.5: RIG-I transfection

When overexpressing RIG-I in ASK-cells, the chaperone GRP78 was significantly up-regulated reaching a peak on day 3 post transfection. This probably accounts for the synthesis of new proteins, in this case the RIG-I where the expression is driven by the CMV promoter, IFNa and Mx.

The expression of all the target genes seems to follow the same pattern with peaks and declines on the same days in both RIG-I and MAVS-transfected ASK-cells which is plausible. The main difference observed with the RIG-I and MAVS-transfected ASK-cells is the stronger induction of IFNa on day 1 post transfection with MAVS-plasmids compared to the induction seen with RIG-I-plasmids on IFNa gene expression on the same day. This can also be observed for the IL-1β gene, where the up-regulation seen on day 1 post transfection is stronger with the MAVS-plasmids compared to the RIG-I-plasmids.

It was investigated whether overexpressing RIG-I in CHSE-cells could confer any kind of protection of the cells against infection with these two pathogenic viruses. From our results it appears that RIG-I overexpression in CHSE-cells may give some protection against infection with IPNV (see Figure 34), but the same could not be observed for the ISA virus as the variation in these data was too large for any conclusion to be drawn. However, although the RIG-I transfection appears to protect the CHSE-cells in the fluorescence microscopy experiment, this was not observed when performing the $TCID_{50}$ titration (data not shown). For the $TCID_{50}$ titration, the situation was reversed, suggesting that MAVS-transfection could protect the cells against IPNV infection (Figure 36 and Figure 37).

When transfecting with RIG-I-GFP, this protein showed an even distribution throughout the cytosol of the cell. However more often than not, the RIG-I-GFP a couple of days after transfection, could be observed as vacuoles in close proximity to the cell nucleus which may suggest a saturation of the ER. Sometimes RIG-I-GFP even showed a co-localization with the nucleus as can be observed in Figure 33 in the right picture. Overexpressing RIG-I may alter the natural distribution of this protein in these cells. Whether this is attributed to the fact that the proteins are overexpressed or that the GFP-tag attached to the RIG-I and MAVS may alter the function and distribution of them, remains unclear. One should also keep in mind that the RIG-I-GFP is not the full-length RIG-I but the N-terminal end fused with the GFP and that this may also contribute to a possible alteration of subcellular distribution.

However, it should be kept in mind that the transfection itself may induce immune responses and alter the transcription of the selected target genes investigated in this thesis, and that in this thesis, Q-PCR was not run on empty plasmid vector transfected cells as a control making the interpretation of the Q-PCR results for transfected cells more difficult to interpret.

In summary our results may indicate that overexpression of RIG-I and MAVS may have some kind of protective effect against IPNV infection, but our results do not confirm this beyond a doubt. Further studies are required to investigate this. For ISAV no conclusive results were obtained regarding MAVS and RIG-I overexpression and if it could confer some protection against ISAV infection. This was due to the difficulties in observing CPE in infected cells with our ISAV4 isolate, after it had been propagated in ASK-cells. Even though the ISAV4 isolate used in this thesis may have been attenuated in some way (either by excessive thawing and refreezing or to many passages), it did replicate in ASK-cells and made the cells present pathological changes like vacuolization in the infected cells and also induced IFNa up-regulation in infected ASK-cells.

6: Concluding remarks and future direction

Due to the significance of viral infection with IPN and ISA and their related mortalities in commercially cultured salmon populations, there is a strong interest aimed toward understanding viral infection in fish and the development of methods including vaccination to combat such outbreaks. Novel vaccines against IPNV and ISAV are needed as the available vaccines on the market do not prove to be sufficiently effective in controlling new disease outbreaks and preventing a carrier state in fish. Knowledge on RLR ligands and the way they activate the innate immune system begins to be exploited and certainly will in the future be important for vaccine development as teleost fish are more dependent on their innate immune system as their adaptive immune response is not as specific as the mammalian system. In this master thesis the viral infections with IPNV and ISAV and the immune responses they induce and also how the overexpression of MAVS and RIG-I could influence the infection progression were covered.

A central issue that remains to be resolved is the intensity of the host response in a specific tissue targeted by viral infection. The fundamental role of the inflammatory response and its involvement in either resolution of viral infection or dysfunctional responses leading to the establishment of asymptomatic carriers or extensive tissue damage leading to a negative outcome is central and should be further investigated in the future. Understanding the complex network of cellular processes and virus-host interactions should aid in identifying new and common targets for the therapeutic intervention of virus infection aiding in producing novel vaccines. This effort must take advantage of the recent developments in functional genomics, bioinformatics and other emerging technologies as suggested by Katze *et al* [73].

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