Thesis submitted for the degree of
Candidata Pharmaciae

Effect of infectious salmon anemia virus (ISAV) infection and poly I:C treatment on expression of different stress related genes in Atlantic salmon (*Salmo salar L.*) cells

By

Annbjørg Ringheim Kvello

Department of Pharmaceutical Biosciences

School of Pharmacy

Faculty of Mathematics and Natural Sciences

University of Oslo, Norway

Oslo, November 2007

ACKNOWLEDGEMENTS

CONTENTS

1 ABBREVIATIONS

ASK	Atlantic salmon kidney
Bcl-2	B-cell lymphoma 2 protein
BSA	Bovine Serum Albumin
Cox-2	Cyclooxygenase 2
CPE	Cytopathic effect
Ct	Treshold cycle
DNA	Deoxyribonucleic acid
ds	Double-stranded
FADD	Fas-assosiated death domain
FBS	Fetal bovine serum
Fip-2	Fourteen Kilodalton Interacting Protein 2
HA	Hemagglutinin
HE	Hemagglutinin esterase
HRP	Horseradish peroxidase
Hsp	Heat shock protein
IAP	Inhibitor of apoptosis
IFN	Interferon
IL	Interleukin
IPNV	Infectious pancreatic necrosis virus
ISA	Infectious salmon anemia
ISAV	Infectious salmon anemia virus
ISG	Interferon stimulated gene
kDa	kilodalton
M1	Matrix protein 1
M2	Matrix protein 2
Mcl-1	Myeloid leukemia differentiation protein-1
mRNA	messenger RNA
NA	Neuramidase
NP	Nucleoprotein
OGFr	Opioid growth factor receptor
PB1	Polymerase protein
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
Pdcd5	Programmed cell death 5
p.i.	Post infection
PKR	Protein kinase-R
Poly I:C	Polyriboinosinic-polyribocytidylic acid
REST	Relative Expression Software Tool
RNA	Ribonucleic acid
RNP	Ribonucleoprotein complex
RT-PCR	Reverse transcription polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SHK	Salmon head kidney
TNF	Tumor necrosis factor
TRAIL	Tumor necrosis factor-related apoptosis inducing ligand

2 ABSTRACT

Infectious salmon anemia virus (ISAV) is a virus causing severe disease in Atlantic salmon (*Salmo salar L.*). Although its structure and pathogenesis is well described, little is known about its effects on the expression of genes related to different stress responses in the host cell. ISAV is a virus that is probably causing apoptosis in Atlantic salmon, but the mechanisms are not yet fully understood. Interferons (IFN) and interferon induced genes (ISG), which is important of the host control of virus infections, is highly expressed after ISAV infection. But it looks like that ISAV has developed mechanisms that help it evade the host immune response.

In this study we analyzed the relative expression of different stress related genes after ISAV infection and poly I:C stimulation in Atlantic salmon kidney cell line (ASK). Poly I:C is widely used as synthetic dsRNA analog *in vivo* and *in vitro* and has also demonstrated to induce antiviral responses in Atlantic salmon. When we compare these two stress situations with each other we may better understand the innate immune responses during viral infections in this commercially important fish species.

As measured by quantitative real-time PCR, both ISAV and poly I:C stimulated cells induced a high up-regulation of interferon-α and interferon induced genes as expected.
In confocal immunofluorescence microscopy it was detected that ISAV still replicates at day 5 post infection, and that it induces a cytophatic effect in ASK cells, indicating that interferons have little antiviral effect on ISAV.

Furthermore, there was observed an up-regulation of different cytokines, stress related genes and even apoptotic related genes, like IAP, both in ISAV infected and poly I:C stimulated ASK cells.

Galectin-9, that may be involved in T-cell apoptosis, was up-regulated in both ISAV infected and poly I:C stimulated cell. The protein expression of galectin-9 was analysed using immunofluorescence microscopy.

The result of this study strongly indicates that different stress related genes, which may be involved in the host immune response is induced after ISAV infection. These results can lead to a better understanding of the pathogenesis of ISAV in Atlantic salmon.

3 INTRODUCTION

3.1 *Orthomyxoviridae*

ISAV is a member of the *orthomyxoviridae*-family. The *Orthomyxoviridae* are a family of RNA viruses that includes five genera: *Influenza virus A, Influenza virus B, Influenza virus C,* the tick-borne *Thogotovirus* and the marine/aquatic *Isavirus.* Orthomyxoviruses are enveloped and contain segmented, linear and negative-sense (complementary to mRNA) single-stranded RNA.

The genomic RNA of negative-strand RNA viruses has to serve two functions; first as a template for synthesis of mRNA and second as a template for synthesis of the antigenome (+) strand. Negative strand RNA viruses encode and package their own RNA-dependent RNA polymerase, but mRNAs are synthesized only after the virus has been uncoated in the infected cell. Viral replication occurs after synthesis of the mRNAs and requires synthesis of viral proteins. The newly synthesized antigenome (+) strand serves as the template for further copies of the (-) strand genomic RNA (Fields et al 1996).

It has been shown that influenza A, influenza B and ISAV contain eight RNA segments;(Mjaaland et al., 1997) influenza C contains only seven segments and the Thogotovirus six segments (Cox et al., 2004, Hagmaier et al., 2003)

The most conserved othomyxovirid protein has been shown to be the polymerase protein (PB1), which makes it a good candidate to evaluate the evolutionary relationships between ISAV and members of the *Orthomyxoviridae.*

The occurrence of consensus regions in the RNA-dependent DNA polymerase has led to the assumption that the sequence similarities may be linked to the existence of a common ancestral genetic element bearing a polymerase function, which emerged only once during the evolution (Krossoy et al., 1999). (Fig 3.1)
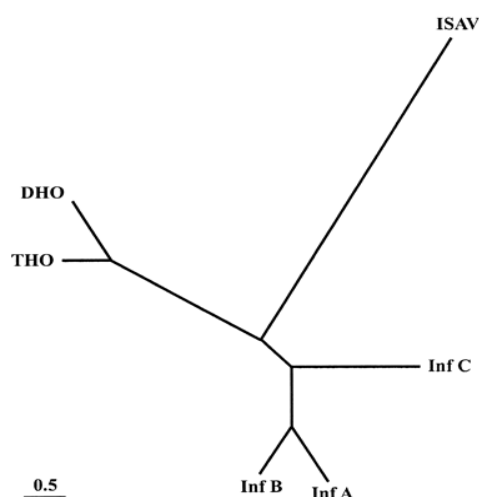
Fig 3.1: Genetic distance tree drawn by the neighbour-joining method. The relationship between the orthomyoviruses based on the PB1 polymerase protein (figure from (Krossoy et al 1999))

Calculations of the distances between the different PB1 proteins indicate that the ISAV is distantly related to the other members of the family, but more closely related to the influenza viruses than to the Thogoto viruses.

3.1.1 Structure and genome organization of orthomyxoviruses

Influenza viruses are part of the family *Orthomyxoviridae*, and can be classified into Influenza A, Influenza B and influenza C. Influenza A are further classified into subtypes based on the antigenicity of their hemgglutinin (HA) and neuraminidase (NA) molecules.

All influenza viruses are characterized by a segmented negative-sense RNA core surrounded by a lipid envelope. The A and B types are distinguished by two integral membrane glycoproteins, HA and NA, that protrude from the virion surface. Within the lipid envelope are the matrix (M1) protein and RNA segments, which are associated with nucleoprotein (NP) and three large polymerase proteins, PA, PB1 and PB2. These polymerase proteins, with NP, are responsible for RNA replication and transcription.

The HA spikes are rod-shaped, whereas the NA spikes resemble mushrooms with slender stalks. (**Fig 3.2)**
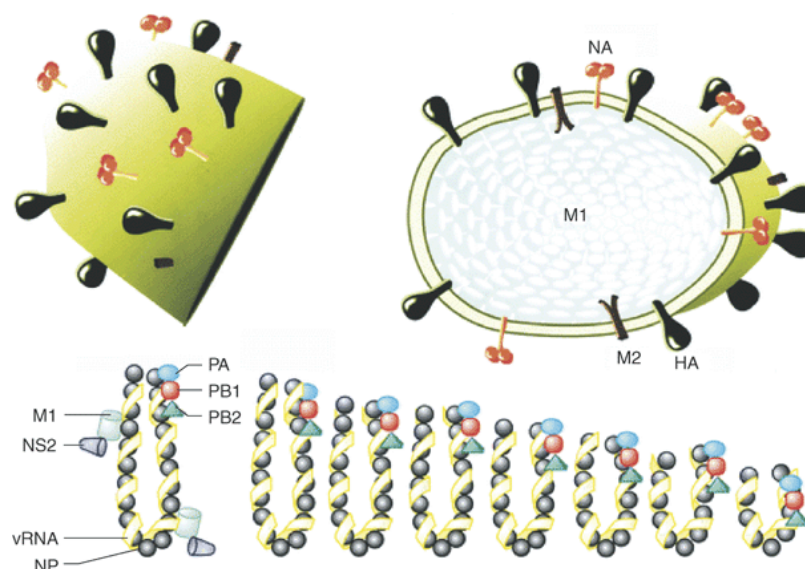
Fig 3.2 Structural features of influenza A virion. Two glycoprotein spikes, hemagglutinin (HA) and neuraminidase (NA) and the M2 protein are embedded in a lipid bilayer derived from the host plasma membrane. The ribonucleoprotein complex (RNP) consists of viral RNA, associated with the nucleoprotein (NP) and three polymerase proteins (PA, PB1, PB2). NS2 (NEP) is associated with RNP, while the M1 protein is associated with both RNP and the viral envelope. Thus, NS1 is the only non-structural protein of influenza A virus. (figure from (Neumann, 2007)

Influenza A virus contains genes for nine structural and one non-structural protein. The polymerase proteins PA, PB1 and PB2 are coded by the largest RNA segments. These three proteins are highly conserved in influenza A viruses. PB1 is required for the initiation and elongation of newly synthesized viral RNA. PB2 recognizes and binds to type I cap structures of cellular mRNA, and is essential for viral mRNA synthesis. The PA protein is an essential component of the viral polymerase complex. It is required for viral RNA replication and may be involved in transcription. Accounting for about 25 % of viral proteins, the HA is distributed evenly on the surface of virions and is responsible for the attachment and subsequent penetration of viruses into cells. NP, the most abundant component of RNP, is a type-specific antigen associated with viral RNA (Neumann, 2007).

The tetrameric NA protein of influenza A viruses is one of two major glycoproteins on the virus surface. Both HA and NA plays a role in host range restriction.

M1 is the most abundant virion protein and a type-specific antigen of influenza viruses. M1 is the major determinant of virus budding; furthermore it determines the morphology of influenza virions.

M2 proteins are thought to function as pH-activated ion channels that permit protons to enter the virion during uncoating, thereby modulating the pH of intracellular compartments. This function is essential for the prevention of acid-induced conformational changes of intracellularly cleaved HAs.

NS1 is the only non-structural protein of influenza A virus, and is produced in abundance during early infection. It binds to double-stranded RNA, preventing the activation of interferon-induced protein kinase R and transcription factors (described later), suggesting a role of this protein in the prevention of interferon-mediated antiviral responses.

NS2 (NEP) likely functions as the viral nuclear export protein by connecting the cellular export machinery with vRNPs through M1 (Neumann, 2007).

3.2 Infectious salmon anemia virus

Infectious salmon anaemia virus (ISAV), an economically important new pathogen in marine aquaculture (salmon farming), is classified in the family *Orthomyxoviridae,(Falk et al., 1997)* genus *Isavirus*. It was first described as a disease entity in juvenile Atlantic salmon in Norway in 1984. Affected fish were found to be lethargic and had severe anemia with characteristic symptoms like exophthalmia, pale gills, ascites, hemorrhagic liver necrosis, renal interstitial hemorrhage and tubular nephrosis (Thorud, 1988). Outside Norway ISAV was recognized during 1997-2000 in salmon farms on the Atlantic coast of Canada, USA, in Scotland and the Faeroes.

There are indications that the gills are the most likely port of entry for ISAV, but the intestinal route cannot be excluded (Mikalsen et al., 2001). Endothelial cells are one of the main target cells for ISAV, but the presence of virus in other cells, like polymorphonuclear leukocytes, has been reported.

ISAV is known to cause overt and fatal systemic infection in farmed Atlantic salmon. 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 differences or environmental factors caused by management practices on salmon farms.(Kibenge et al., 2004)

ISAV may infect and replicate in sea trout, rainbow trout, eels, herring (*Clupea harengus)* and Artic char (*Salvelinus alpinus)*, resulting in asymptomatic, probably life-long, carrier status of the virus (Kibenge et al., 2004).

ISAV does not infect human or other mammals because the virus is inactivated at pH values below 4.5 and does not replicate *in vitro* at temperatures of 25 °C or above. (Falk et al., 1997) The optimum growth temperature for ISAV in fish cell lines is 10-15 °C (Dannevig et al., 1995) (Falk et al., 1997).

Before 1995, the isolation and propagation of ISAV *in vitro* was not possible because the continuous fish cell lines available at the time did not appear to support replication of the virus. In 1995 a continuous cell line, SHK-1, was established from a culture of Atlantic salmon head kidney leukocytes. The SHK-1 cell line allowed replication of ISAV with development of cytopathic effects (CPE) (Dannevig et al., 1995). Additional cell lines that support the replication of ISAV have later been developed and are, like the SHK-1 cell line, able to produce CPE. The Atlantic salmon kidney (ASK) cell line was also developed from Atlantic salmon head kidney tissue (Devold et al., 2000). Comparison of the SHK-1 and ASK

cell lines indicated that the ASK cell line was highly useful in a diagnostic laboratory setting based on its ability to adapt to standard cell culture routines, ease of maintenance, and the rapid and distinct CPE induced by ISAV (Rolland et al., 2005).

3.2.1 Structure and genome organization of ISAV

ISAV shares several morphological, biochemical and physiochemical features with influenza viruses. It is the only known species of the genus *Isavirus*, one of the five genera of the family *Orthomyxoviridae*, as already described. Viruses in the genus *Isavirus* are enveloped particles 90-140 nm in diameter with 13-15 nm long mushroom-shaped surface projections consisting of a combined reseptor-binding hemagglutinin and receptor-destroying enzyme activity that has been demonstrated to be an esterase, hence recently designated HE (Falk et al., 2004).

The genome is composed of eight segments of linear, single-stranded negative sense RNA ranging in length from 1.0 to 2.4 kb with a total molecular size of approximately 14.3 kb (Clouthier et al., 2002). All the eight RNA segments of ISAV have been sequenced. The order of the genome segments encoding the proteins in ISAV appears to differ from those of influenza viruses (Table 3.1).

Table 3.1: Comparison of genome coding assignment for Isavirus and Influenzavirus A

Genome segment	Isavirus	Influenzavirus A
1	PB2 (84 kDa)	PB1 (96 kDa)
2	PB1 (84 kDa)	PB2 (87 kDa)
3	NP (77 kDa)	PA (85 kDa)
4	PA (71 kDa)	HA$_1$ (48 kDa), HA$_2$ (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 (9,5 kDa)	M1 (25 kDa), M2 (15 kDa)
8	M1 (24 kDa), NS1 (16 kDa)	NS1 (25 kDa), NS2 (12 kDa)

Gene products: PB2, PB1 and PA, polymerase; NP, nucleoprotein; HA, hemagglutinin; gp50, an envelope protein, that may be a fusion-protein in *Isavirus*; HE, hemagglutinin esterase in Isavirus; NA, neuraminidase; M1, matrix protein; 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 (NEP). (Kibenge et al., 2004)

The proteins comprising the polymerase heterotrimer are analogous to influenza virus. Segment 1 encodes the protein that is thought to be the PB2 protein of ISAV (Snow et al., 2003b). Segment 2 encodes the PB1 protein, which is the most conserved protein of orthomyxovirus; while the third polymerase protein PA, is encoded by segment 4 (Ritchie et al., 2001) (Snow and Cunningham, 2001). Along with the polymerase proteins, the nucleoprotein is encoded by segment 3 (Ritchie et al., 2001). ISAV contains two surface glycoproteins encoded by segment 5, gp50 (47 kDa) and 6 (42 kDa). gp50 is an envelope protein that has been shown to be a membrane fusion protein which requires proteolytic cleavage to induce fusion (Aspehaug et al., 2005), while the 42 kDa protein encodes the hemagglutinin-esterase (Krossoy et al., 2001) (Rimstad et al., 2001). Segments 7 and 8 have two overlapping ORFs. ISAV genomic segment 7 was suggested to share a similar coding strategy with segment 7 of influenza A virus, encoding two proteins. But it has been shown that ISAV segment 7 encodes 3 molecules with estimated molecular masses of 32, 18 and 9.5 kDa. The 32 kDa protein is a structural protein, the 18-kDa protein is identified as the putative ISAV nuclear export protein, while the function of the 9.5 kDa protein is not known (Kibenge et al., 2007). It is only speculated that segment 8 encodes the matrix protein (Bierin et al., 2002). (Table 3.2)

Thus, the ISAV genome encodes at least ten proteins, of which nine are structural and one is non-structural. (Kibenge et al., 2004).

Table 3.2: RNA-segments to ISAV

Segment	Segment Length (kb)	Encoded protein	Predicted Molecular Mass (kDa)
1	2.4	Polymerase (PB2)	84
2	2.4	Polymerase (PB1)	84
3	2.2	Nucleoprotein (NP)	77
4	1.9	Polymerase (PA)	71
5	1.6	Envelope protein (gp50)	47
6	1.5	Hemagglutinin esterase (HE)	42
7	1.3	p32, nuclear export protein(NEP), p11	32,18,9.5
8	1.0	Matrix protein (M1), non-structural protein 1 (NS1)	24,16

3.3 Apoptosis and virus infection

There are two major modes of cell death, i.e., necrosis and apoptosis, and these differ both morphologically and biochemically. Necrosis is characterized by the swelling of mitochondria, early rupture of the plasma membrane, dispersed chromatin and early destruction of the intact structure of the cell. Apoptosis is characterized morphologically by cell shrinkage and hyperchromatic nuclear fragments and biochemically by chromatin cleavage into nucleosomal oligomers. The cell will shrink and condense into multiple small membrane-bound "apoptotic-bodies" which can be targets for phagocytes. This removes apoptotic cells without leaking the cytoplasmic contents into the intercellular space, and this will minimize tissue inflammation (Mullauer, 2006).

Apoptosis has now been documented for many cell types in addition to lymphocytes. It can occur in response to stimuli ranging from toxic substances to hormone and cytokine addition or withdrawal and, most importantly, virus infections (Hinshaw et al., 1994).

The original definition of apoptosis states that this form of cell death does not make an inflammatory response. However, recently this class definition has been revised as apoptosis in certain situations, such as pathogen invasion, can induce an inflammatory response, which may promote the activation of an immune response (Restifo, 2000).

Apoptosis is characterized by several morphological changes, and can be triggered via one of several pathways. It can activate the protein kinase/ phosphatase cascade or the release of secondary messengers, which act as positive or negative transcription factors for specific genes.

Another common event in most of these pathways is the activation of a group of proteases called caspases. Caspases can be separated into two groups, the initiator (capasese-8, -9, -10 and -12) and the effector (caspase-1, -3, -6 and -7).

Influenza virus can induce apoptosis in different cell types in the latest stages of infection both *in vivo* and *in vitro*, but the mechanism is still unclear. One of the major problems is the large number of cell types being studied, because the apoptotic response can vary from different cells and activation stimulus. Furthermore, the initiation of one pathway can induce multiples signal transduction cascades through feedback loops, but the final outcome of the apoptotic response appears to be universal. (**Fig 3.3**)

Fig 3.3: Apoptosis signalling pathways induced by influenza virus infection. Upon engagement of Fas ligand (FasL) a death signal is transmitted from Fas by the recruitment of FADD through the interactions of their death domains (DD). FADD binding is followed by pro-caspase-8 binding, to form the death-inducing signalling complex (DISC). This results in the activation of caspase 8, which subsequently leads to the initiation of a caspase cascade and ultimately the activation of death substrates. Caspase-8 also acts on the mitochondria causing a permeability transition (PT) pore. This results in the release of cytochrome c, which in association with Apaf-1 activates caspase-9 family members, thus enhancing the caspase cascade. Bcl-2 inhibits the release of cytochrome c from the mitochondria but is itself inhibited by Bax. Apoptosis inducing factor (AIF) is also released from dysfunctional mitochondria. AIF binds DNA initiating chromatin condensation. The activation of protein kinase (PK)-R by double stranded (ds) RNA can also activate caspase-8, by a Fas-independent mechanism, leading to the activation of caspase-9. In addition, PKR activates Nuclear Factor (NF)-jB leading to up-regulation of pro-apoptotic gene expression. NF-jB can also be indirectly activated by reactive oxygen species (ROS) and free radicals produced during virus infection. ROS and free radicals also act on the mitochondria causing changes in membrane potential and the release of cytochrome c. Endoplasmic reticulum (ER) stress due to the overproduction of viral glycoproteins activates apoptosis signal-regulating kinase (ASK)-1, which together with tumour necrosis factor receptor-associated protein (TRAF)-2 binds to interferon response element-1 resulting in the up-regulation of pro-apoptotic genes. PKR may also directly activate ASK-1. Another apoptotic pathway involves the activation of TGF-b. TGFb initiates a signalling cascade leading to the activation of c-Jun N-terminal kinas (JNK) or stress activated protein kinase (SAPK) again resulting in the activation of transcription factors and up-regulation of pro-apoptotic gene expression. Different virus protein can also be involved in apoptosis. (Figure from (Brydon, 2005)

Protein kinase R (PKR) is a key regulatory component in many apoptotic pathways and is induced by IFN and activated by dsRNA. Activated PKR has been detected in a number of different cell types infected with influenza virus (Takizawa et al., 1996, Balachandran et al., 1998), and leads to several downstream events like phosphorylation of eukaryotic initiation factor (eIF)-2,(inhibit protein synthesis) activation of transcription factor NF-κB and transcriptional induction of pro-apoptotic genes like those coding Fas, p53 and Bax.

Active PKR can also lead to a recruitment of caspase-8 via the cytoplasmic protein Fas associated domain (FADD), which leads to initiation of the caspase cascade. In Madin-Darby canine kidney (MDCK) cells, the influenza virus-induced apoptosis correlate with an up-regulation of Fas, in addition caspase-8 and caspase-3 are activated. This suggest that virus-induced apoptosis is, at least in part, mediated via the formation of a FADD/caspase-8 complex by PKR, which activates the caspase cascade independently of Fas (Balachandran et al., 1998). But it has also been indicated that Fas may be involved in viral induced apoptosis, perhaps as a secondary mechanism.

Another apoptotic pathway involves the activation of TGF-β. TGF-β initiates a signalling cascade leading to the activation of c-Jun N-terminal kinases (JNK) or stress activated protein kinase (SAPK) resulting in activation of transcription factors and up-regulation of pro-apoptotic gene expression.

Viral neuraminidase (NA) activates latent TGF-β, and influenza virus-induced apoptosis of MDCK cells is partially inhibited by TGF-β-specific antibodies, which indicates the important role of TGF-β in apoptosis (Schultz-Cherry and Hinshaw, 1996). TGF-β activation has also been in apoptosis of lymphocytes.

Recent studies have indicated that caspase-8 activation is the predominant apoptotic pathway in human bronchiolar cells, but it has been shown that a second apoptotic pathway has been indicated, the apoptosis signal-regulating kinase (ASK)-1 pathway (Maruoka et al., 2003).

Endoplasmic reticulum (ER) stress due to the overproduction of viral glycoproteins activates ASK-1, which together with tumor necrosis factor receptor-associated protein (TRAF)-2 binds to interferon response element-1 resulting in the up-regulation of pro-apoptotic genes (Maruoka et al., 2003). The activation of PKR may also potentiate this system, by direct activation of ASK-1.

Studies using the human embryonic kidney cell line have shown that M1, HA, or NP generates reactive oxygen species (ROS), which leads to IκB kinase expression and ultimately NF-κB activation (Flory et al., 2000).

Many of the apoptotic pathways described above result in the activation of transcription factors such as AP-1 and NF-κB, which are known to regulate the expression of several cytokines and chemokines. Thus, the activation of these transcription factors provides a direct link between apoptosis, cytokine expression and inflammation (Brydon et al., 2005).

An intrinsic apoptosis pathway focuses on mitochondria and is triggered by various types of cellular stress that lead to a change in the permeability of the outer mitochondrial

proteins into the cytosol. Cytochrome c plays a key role, and it will bind to the cytosolic adaptor molecule Apaf-1 and induce its oligomerization (Kroemer and Reed, 2000). Oligomerized Apaf-1 activates caspase-9, which activates a cascade of caspase reactions.

The mitochondrial branch of apoptosis signalling is regulated by bcl-2 and its homologs. Bcl-2 is an antiapoptotic protein that is on the cytoplasmic face of the mitochondrial outer membrane and inhibits apoptosis by blocking the release of cytochrome c. It is the prototype of a family of related proteins with either antiapoptotic (Bcl-2, Bcl-X$_L$) or proapoptotic (noxa, Bid, Bax, Bcl-X$_s$, Bak and Bad) functions. Pro- and antiapoptotic Bcl-2 family members and their relative concentration determine the susceptibility to apoptotic stimuli (Gross et al., 1999).

A second intrinsic apoptosis pathway features the nuclear protein p53 at center stage. The molecule links cell damage to cell cycle arrest and apoptosis. It is activated in response to DNA damage and arrest cells with damaged DNA in the cell cycle. If DNA damage is severe, and dependent on cell type and the state of oncogene activity of a cell, p53 initiates apoptosis, partially by inducing the transcription of proapoptotic genes like Bcl-2 associated X protein (Bax) (Vogelstein et al., 2000).

The role of various viral proteins during influenza virus-induced apoptosis has also been studied. It has been shown that NS1 (non-structural) protein is capable of inducing apoptosis when expressed in cell cultures (Schultz-Cherry et al., 2001). Other studies, however, suggest that NS1 also possesses anti-apoptotic potential (Zhirnov et al., 2002a).

As already mentioned earlier, NA can activate latent TGF-β, a broad inducer of apoptosis.

Interaction of M1 (matrix) protein of influenza A virus with cellular caspase-8 suggests that M1 protein may have a role in virus-induced apoptosis (Zhirnov et al., 2002b).

A new influenza virus gene product, PB-F2 (produced by a +1 reading frame in the viral RNA segment that encodes polymerase subunit PB1) has also been shown to play an important role in apoptosis (Chen et al., 2001a). Thus, influenza viruses may have multiple mechanisms that contribute to the induction of apoptosis in host cells.

ISAV-induced apoptosis in SHK-1 cells occurs via the caspase-activation pathway, but may not involve activation of caspase-3. It has been shown that ISAV proteins, particularly the protein encoded by RNA segment 7 ORF2, have the potential to bind caspase-8 specifically, which might have implications in ISAV-induced apoptosis (Joseph et al., 2004).

3.4 Interferon-response and virus infection

When a cell becomes infected with a virus it responds by making cytokines called type I interferons. The main effects of interferon are to interfere with viral replication in the infected cell, and to signal neighboring uninfected cells that they too should prepare for a viral infection. Further effects are to alert cells of the immune system that an infection is started. Virtually all cell types can make interferon and its cell-surface receptor, consequently making them ready to thwart a virus-infection. There are many different forms of type I interferons. The most important ones are interferon-β and interferon-α.

Interferon response can lead to an induction of cellular proteins that interfere with viral genome replication. As well as interfering with viral replication, interferon also induces cellular changes that make the infected cell more likely to be attacked by killer lymphocytes (Natural killer cells)(Parham, 2005).

The type I interferon (IFN) system plays a major role in the innate antiviral immune system of vertebrates. In most mammalian cells the IFN system is activated by recognition of viral dsRNA (replicative intermediate) through intracellular receptors, which include the two RNA helicases RIG-I and MDA5 and the endosomal located Toll-like receptor 3 (TLR3) (Kato et al., 2006, Alexopoulou et al., 2001). Binding of dsRNA to RIG-1, MDA5 or TLR3 leads to activation of the transcription factors NF-κB and IRF-3(interferon-response factor 3), resulting in transcription of the IFN-β gene.

The secreted type I IFNs act like alarm proteins, which will bind to specific cell surface receptors associated with the Jak/STAT signalling pathway. Activation of this pathway results in induction of expression of interferon stimulated genes (ISGs), some of which encode antiviral proteins including Mx and double-stranded RNA activated protein kinase (PKR) (Fig 3.4).
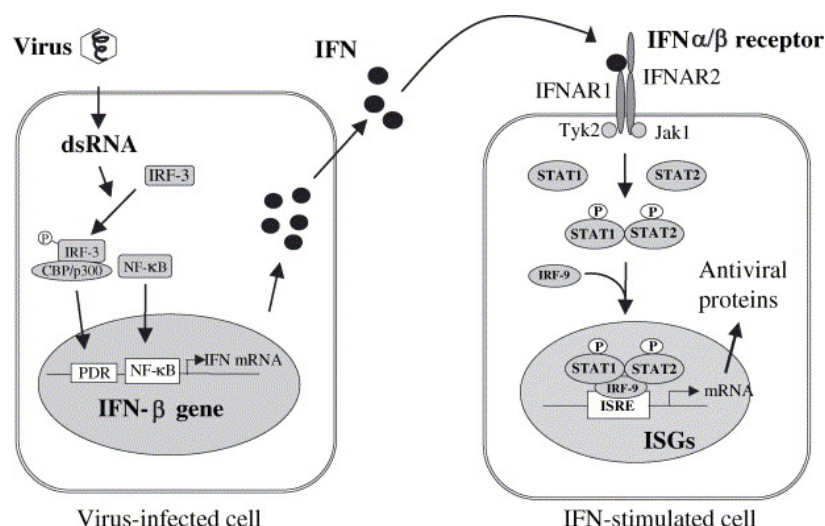
Fig 3.4: An illustration of the 2-step signal pathway of type I IFNs represented by IFN-β, which is the first IFN to be produced during virus infection. (Figure from (Robertsen, 2006)

The cloning of IFN and several typical ISGs from Atlantic salmon and other fish species has established that teleost fish possess a type I IFN system similar to mammals (Robertsen, 2006). Both Mx and ISG15 have been cloned from salmon. Antiviral activity of Mx protein is well documented in mammals (Haller et al., 2007), and has recently also been demonstrated against IPNV in Atlantic salmon. Human and mouse Mx confer resistance against the orthomyxoviruses influenza A and B (Haller et al., 2007), and human Mx in addition possesses antiviral activity against a variety of other viruses. The antiviral mechanism of Mx protein is still uncertain, but it has been hypothesized to interfere with viral replication by preventing intracellular trafficking of viral nucleocapsids (Haller et al., 1998).

ISG15 is one of the earliest and most predominant proteins to be induced in mammals following IFN-α/β stimulation. This implies that ISG15 has an important function in the IFN system. The function of ISG15 is not yet understood, but it has been shown that it has a similar function as ubiquitin. ISG15 conjugates to cellular proteins in both human, goldfish and in Atlantic salmon (Rokenes et al., 2007). Some evidence for antiviral activity of ISG15 has also been presented. Influenza B NS1 protein has been shown to bind specifically to human ISG15 and block the conjugation to cellular proteins, a phenomenon that supports an antiviral role of ISG15. Binding of Atlantic salmon ISG15 to an ISAV protein has been reported, but the functional relevance to this is not known (Rokenes et al., 2007).

Both poly I:C, an synthetic dsRNA, and ISAV induce a strong IFN response, but with no inhibition of virus replication This can indicate that ISAV has a mechanism that can counteract this immune-response (Kileng et al., 2007).

3.5 Apoptotic and immunological genes analysed

In this work we have studied a number of genes that are involved in the innate immune-response of the host cell after an ISAV-infection. Some of these genes were identified after microarray analysis of ISAV infection, and others by literature survey of articles describing different genes that can be involved in stress responses in fish. Based on this, we have compared the effects of poly:IC stimulation and ISAV infection on gene expression in a cell line from Atlantic salmon.

Interferon-α

The type I interferon (IFN)-1 plays a major role in the innate antiviral immune system of vertebrates and mammals. Interferon is a cytokine expressed in cells after virus-infection. The main effect of interferon is to interfere with viral replication in the infected cell, and to signal neighboring uninfected cells that they too should prepare for a viral infection (more details is discussed in earlier chapter).

MX

Mx proteins are key components of the antiviral state induced by interferons in many species. They belong to the class of dynamin-like large guanosine triphosphatases (GTPases) known to be involved in intracellular vesicle trafficking and organelle homeostasis. A unique property of some Mx GTPases is their antiviral activity against a wide range of RNA viruses, including influenza viruses, member of the orthomyxoviruses, and members of the bunyavirus family. These viruses are inhibited at an early stage in their life cycle, soon after host cell entry and before genome amplification. The mouse Mx1 GTPase accumulates in the cell nucleus where it associates with components of the PML nuclear bodies and inhibits influenza and Thogoto viruses known to replicate in the nucleus. The human MxA GTPase accumulates in the cytoplasm and is partly associated with the endoplasmic reticulum.

In the case of Thogoto virus (orthomyxovirus), MxA recognizes the viral nucleoprotein and prevents the incoming viral nucleocapsids from being transported into the nucleus, the site of viral transcription and replication. In general, Mx GTPases appear to detect viral infection by sensing nucleocapsid-like structures. As a consequence, these viral components are trapped and sorted to locations where they become unavailable for the generation of new virus particles (Haller et al., 2007).

Interferon stimulated gene 15

Type I interferons (IFNs) play an essential role in the host response to viral infection through the induction of numerous IFN-stimulated genes (ISGs), including important antiviral molecules such as Mx, iNOS and IFN-stimulated gene 15 (ISG15). ISG15 is an ubiquitin homolog that is rapidly up-regulated after viral infection, and it conjugates to a wide array of host proteins.

IFNs are the most important early defence against acute virus infection. ISG15 is an important host IFN-induced antiviral protein that functions *in vivo* against several important human pathogens.

The antiviral activity of ISG15 may be the result of its cytokine activity or its ability to conjugate to target proteins or both. Human ISG15 is reported to stimulate natural killer cell proliferation, IFN-γ production, neutrophil recruitment, and the maturation of dendritic cells (Lenschow et al., 2007).

Functional studies of the fish ISG15 have hardly been carried out, however, probably due to the lack of specific antibodies against them. The mechanism of IFN mediated antiviral action in fish is poorly understood. There has been described an ISG15-orthologue in Atlantic salmon with characteristic features of ISG15 (AsISG15), and it has been suggested that AsISG15 has a role in the antiviral interferon response of Atlantic salmon (Rokenes et al., 2007).

It has also been shown that ISAV induces the IFN system in Atlantic salmon cells, but without being inhibited (Jensen and Robertsen, 2002).

Opioid growth factor receptor 1

Opioid growth factor receptor (OGFr) is an immune relevant gen.
Opioid peptides act as growth factors in neural and non-neural cells and tissues, in addition to serving for neurotransmission/neuromodulation in the nervous system. The native opioid growth factor (OGF), (Met5)-enkephalin, is a tonic inhibitory peptide that plays a role in cell proliferation and tissue organization during development, cancer, cellular renewal, wound healing, and angiogenesis. OGF action is mediated by a receptor mechanism. Subcellular fractionation studies show that the receptor for OGF (OGFr) is an integral membrane protein associated with the nucleus. Using antibodies generated to a binding fragment of OGFr, this receptor has been cloned and sequenced in human, rat, and mouse (Zagon et al., 2002).

Both OGF and its receptor have been localized by immunocytochemistry in human and mouse skin (Zagon et al., 1996). The growth factor and its receptor are involved in

renewal of epithelium in murine tongue (Zagon et al., 1994) and rabbit cornea following injury (Zagon et al., 1998). Detailed investigation of the opioid factor receptor-mediated mechanism in wound healing was performed by Wilson et al., in 2000. Blockage of the OGFr by administration of opioid antagonist (naltroxene) resulted in increased DNA synthesis and proliferation of the basal cells in epidermis, whereas administration of an opioid peptide markedly suppressed basal cell proliferation. The salmon partial ORF shares 51 % and 62 % similarity with the human isoform. In a study by (Matejusova et al., 2006) it was found that OGFr1 may have a potential involvement in the depletion of mucous cells during prolonged and heavy G. salaries infection, via suppression of DNA synthesis and profound decrease in basal cell proliferation, but the effect was not universal.

Interleulkin-1β

Interleukin is a pro-inflammatory cytokine that is activated after an infection. Like TNFα it facilitates entry of neutrophils, NK cells, and other effectors into infected areas by inducing changes in the endothelial cells of the local blood vessels. It also activates vascular endothelium and lymphocytes in human cells.

Tumor necrosis factor α

Tumor necrosis factor alpha is a pro-inflammatory cytokine. Tumor necrosis factors (TNFs) are pleiotropic cytokines implicated in inflammation, apoptosis, cell proliferation, and a general stimulation of the immune system (Savan et al., 2005). TNFα activates vascular endothelium and increases vascular permeability, which leads to increased entry of IgG, complement, and cells to tissues and increased fluid drainage to lymph nodes(in humans) (Parham, 2005).

TNFα is referred to as an inflammatory cytokine that can directly stimulate cutaneous inflammation, and is induced after mechanical wounding (Cavaillon, 2001).

Heat shock protein 90

Heat shock protein 90 is a survival signalling chaperone, and it has also have been shown to have a proapoptotic role in cancer therapy. (Nieto-Miguel et al., 2007)

Efficient transcription and replication of the influenza virus genome is dependent upon host-derived factors. In a study on influenza-virus, hsp90 has been identified as one of the host factors that stimulate viral RNA polymerase activity. Hsp90 interacted with the PB2 subunit of the viral RNA polymerase through the amino-terminal chaperone domain and the middle region containing a highly acidic domain. The acidic middle region was also responsible for its stimulatory activity. It was found that a portion of Hsp90 is re-localized to the cell nucleus after viral infection. A PB2 fragment containing an Hsp90 binding domain inhibited viral gene expression in a dominant-negative manner. These results suggest that Hsp90 is a host factor for the influenza virus RNA polymerase (Momose et al., 2002).

Heat shock protein 70

Stress or heat shock proteins (HSPs) are remarkably conserved in all living organisms. Their expression is induced in response to a variety of physiological and environmental insults. In the cytosol these proteins play an essential role as molecular chaperones by assisting the correct folding of stress-accumulated misfolded proteins, preventing protein aggregation, transport of proteins, and supporting antigen processing and presentation. Following stress, intracellularly located HSPs fulfil protective functions and thus prevent lethal damage. In contrast, membrane-bound or extracellularly located HSPs act as danger signals and elicit immune responses mediated either by the adaptive or innate immune system. Here, HSPs act as carriers for immunogenic peptides, induce cytokine release or provide recognition sites for natural killer (NK) cells (Multhoff, 2007).

Peptides bound or linked to heat-shock proteins (HSPs) of microbial or mammalian origin have been shown to elicit potent antigen-specific immunity. Some members of the HSP family, such as hsp60, hsp70, hsp90 and gp96, are able also to stimulate cells of the innate immune system directly and thus, act as 'danger'-signaling molecules. This effect is independent of HSP-associated peptides and, in many respects, resembles the effect of lipopolysaccharide (LPS) (Wallin et al., 2002).

Cycloxygenase 2

The influence of COX-2 within the immune system is via synthesis of prostaglandins (PG) that have a variety of functions, but are important in all stages of inflammation. It is well known that PGs can affect cytokine production during an inflammatory response. The roles of PGs in the inflammatory response of mammals include vasodilatation and increased vascular permeability by interaction with histamine and bradykinin, and down regulation of leukocyte functions by reduction of the respiratory burst, lymphocyte proliferation and antibody production. Defiency of Cox-2 results in reduced inflammation and proinflammatory cytokine release, reduced morbidity, and despite higher viral titers, enhanced survival after an Influenca A virus infection (Carey et al., 2005). A cox2-like gene has been cloned in Atlantic salmon (Ingerslev et al., 2006).

Fourteen Kilodalton Interacting Protein2

FIP (Fourteen Kilodalton Interacting Protein) 2 is thought to have a role in tumor necrosis factor α (TNFα) cytolysis and in cellular remodelling. The E3 14.7 kDa protein inhibits TNF-α induced cell apoptosis. Binding of the E3 protein by FIP2 seems to prevent this inhibition and reverse anti-apoptotic effect of the viral proteins. FIP2 shares similarity with the protein FIP3, which is involved in cell cytolysis and NF-kB inhibition.

Additionally, FIP2 may also play a role in signal transduction, vesicular trafficking, and cell morphogenesis. FIPs involvement in cell cytolysis/apoptotic pathways may be modified by binding of anti-apoptotic proteins to its carboxy end. FIP2 may act to enhance or regulate apoptosis by sequestering anti-apoptotic proteins during cell cytolytic events, rather than playing a direct role in activation of apoptosis, as has been previously suggested (Collins et al., 2007).

Galectin-9

Galectins are a family of soluble β-galactoside-binding animal lectins that modulate cell-to-cell adhesion and cell-to-extracellular matrix interactions and play a role in tumor progression, pre-mRNA splicing, and apoptosis.

In mammals, 15 galectins have been studied, all containing conserved carbohydrate-recognition domains (Hsu et al., 2006).

Galectin members are localized on the cell membrane, in the cytoplasm and in the nucleus of cells, but also secreted forms exists (Hirashima et al., 2004).

Galectin-9 (Gal-9) is an integral membrane protein that exists as two isoforms, a long form and a short form. Galectin-9 induces the chemotaxis of eosinophils, inhibits the function of Th1 cells via binding to T-cell receptor and mucin-domain-containing molecule-3 (TIM-3), and promotes maturation of human monocyte-derived dendritic cells.(Ishikawa et al., 2004) Studies have revealed that Gal-9 induces the apoptosis via the calcium-calpain-caspase-1 pathway that is similar to that of the glucocorticoid pathway (Hirashima et al., 2004).

Cells treated with galectin-9 demonstrated activation through intracellular Ca^{2+} release, resulting in cell aggregation and Th1 cell death by both apoptosis and necrosis (Hsu et al., 2006).

Gal-9 plays a role not only in accumulation but also activation of eosinophils in experimental allergic models and human allergic patients, because Gal-9 induces eosinophil chemoattraction in vitro and in vivo and activates eosinophils in many aspects. Galectins has a variety of biological functions, such as cell aggregation and adhesion, proliferation, cell death, and modulation of inflammation. Gal-9 expression can be up-regulated by IL-1β and IFN-γ (Hirashima et al., 2004).

In some cases a galectin can either promote or suppress cell growth, depending on the cell types and doses used. Galectin-3 is the only member known so far to inhibit apoptosis, while galectin-1, -7 and -9 promote this cellular process. Galectins can act either extracellularly or intracellularly to exert effects on cell growth and apoptosis (Yang and Liu, 2003).
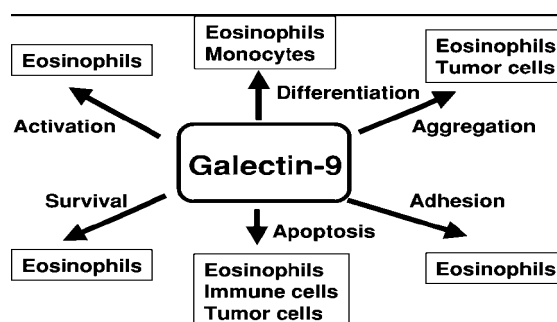

Fig.3.5: Multifunctions of Gal-9 on various cells.
(Figure taken from Hirashima et al., 2004)

Transaldolase-1

Transaldolase 1 is a key enzyme of the reversible nonoxidative branch of the pentose phosphate pathway providing ribose-5-phosphate for nucleic acid synthesis and NADPH for lipid biosynthesis. This pathway can also maintain glutathione at a reduced state and thus protect cellular integrity from reactive oxygen intermediates (ROIs). Formation of ROIs has been implicated in certain types of apoptotic cell death. Transaldolase has a role in regulating the two branches of the pentose phosphate pathway and its overall output as measured by GSH production and thus influences sensitivity to cell death signals (Banki et al., 1996).

Protein p62

Ubiquitin-binding protein p62 is a widely expressed cytoplasmic protein of unclear function. The ability of p62 to bind noncovalently to ubiquitin and to several signalling proteins suggests that p62 may play a regulatory role connected to the ubiquitin system. p62 may have a regulatory function by connecting signal transduction to ubiquitin-mediated proteolysis. It has been shown that p62 up-regulation appears to be a common event in neuronal apoptosis. It has also been suggested that induction of p62 expression by proteasomal inhibitors may be a response to elevated levels of ubiquitinated proteins, possibly constituting a protective mechanism (Kuusisto et al., 2001).

The most well-described and extensively studied function of p62 is its role as a scaffold for selective activation of transcription factor NF-κB (Geetha and Wooten, 2002).

Protein p53

In general, tumor suppressor protein p53 is an essential component of an emergency stress response that prevents the growth and survival of damaged or abnormal cells. Various stresses, like viral infection, increase p53 transcriptional activity, which induces the expression of genes involved in cell cycle arrest and apoptosis. The induction of apoptotic cell death is a hallmark of influenza virus infection. In a study on influenza virus, it was reported that the tumor suppressor protein p53 is a common cellular pathway leading to influenza virus-induced cell death. Surprisingly, inhibiting p53 activity led to elevated virus replication. This may be due to the decrease in interferon signaling in p53-deficient cells, suggesting that functional p53 is involved in the interferon response to influenza infection. This study demonstrates that p53 is involved in influenza virus-induced cell death and that inhibiting p53 leads to increased viral titers, potentially through modulation of the interferon response (Turpin et al., 2005).

p53 can directly activate the proapoptotic Bcl-2 protein Bax in the absence of other proteins to permeabilize mitochondria and engage the apoptotic program. p53 can also release both proapoptotic multidomain proteins and BH3-only proteins (Proapoptotic Bcl-2 family proteins that share only the third Bcl-2 homology domain (BH3)) that were sequestered by Bcl-X_L. When p53 accumulates in the cytosol, it can function analogously to the BH3-only subset of proapoptotic Bcl-2 proteins to activate Bax and trigger apoptosis (Chipuk et al., 2004).

Nf-κB-inhibitor-α-3

NF-κB inhibitor alpha-3 inhibits NF-κB by complexing with and trapping it in the cytoplasm. It may be involved in regulation of transcriptional responses to NF-κB, including cell adhesion, immune and proinflammatory responses, apoptosis, differentiation and growth.

NF-κB is a transcription factor, and activation of NF-κB is important in infections by viral pathogens including influenza viruses. Because gene expression of many proinflammatory and antiviral cytokines is controlled by this factor, the concept emerged that NF-κB and its upstream regulator IκB kinase are essential components of the innate antiviral immune response to infectious pathogens. In contrast to this common view it is reported that NF-κB activity promotes efficient influenza virus production. On a molecular level this is due to NF-κB dependent viral induction of the proapoptotic factors tumor necrosis factor-related apoptosis-inducting ligand (TRAIL) and FasL, which enhance virus propagation in an autocrine and paracrine fashion. Thus, NF-κB acts both proapoptotically and provirally in the context of an influenza virus infection (Wurzer et al., 2004).

NF-κB co-regulates one of the most important antiviral gene expression events, the transcriptional induction of IFN-β. IFN-β is one of the first antiviral cytokines to be expressed upon virus infection.

Another mode of NF-κB interference with virus propagation is through its capability to regulate apoptosis. NF-κB is mainly regarded as a survival factor by up-regulation genes encoding for antiapoptotic proteins, such as Bcl-X_L. NF-κB has also been reported to act proapoptotically under certain conditions (Wurzer et al., 2004).

Inhibitor of Apoptosis Protein

Inhibitor of Apoptosis (IAP) proteins first discovered in baculoviruses, were shown to be involved in suppressing the host cell death response to viral infection.

Caspase inhibition is one possible mechanism for IAPs suppression of apoptosis

It has been shown that different IAPs, like IAP-1, can bind and potently inhibit caspase 3, 7 and 9, but not caspase 1, 6, 8 or 10 (Deveraux et al., 1997,1998).
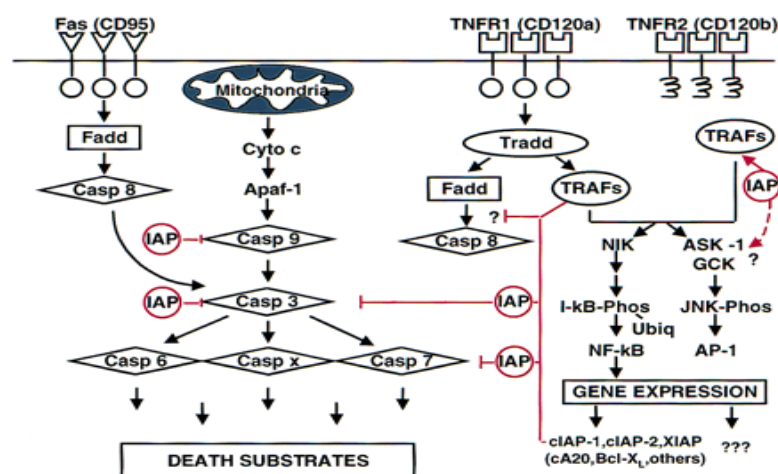
Fig. 4.6: The role of IAP in apoptosis (fig. from (Deveraux, 1999)

In addition to suppressing apoptotic cell death, IAPs are involved in an increasing number of other cellular functions, including cell cycle and intracellular transduction.

Caspase-3

Caspases are a family of cysteine proteases that play important roles in regulating apoptosis. Caspase-3 is one of three highly homologous caspases (caspase-3, -6 and -7) that form the execution subfamily (effector caspases). It has been shown that depletion of caspase-3 in a cell-free apoptotic system inhibited most of the downstream events, including various substrate cleavages, DNA fragmentation, chromatin marginalization, etc, whereas elimination of either caspase-6 or-7 had no effect. Thus, caspase-3 is important for the execution of certain, but not all, specific downstream events of apoptosis (Degterev et al., 2003).

Programmed cell death protein 5

The programmed cell death 5 (PDCD5) protein is a recently discovered protein related to regulation of cell apoptosis. PDCD5 is also designated TF-1 cell apoptosis related gene-19 (TFAR19), and is a novel gene cloned from TF-1 cells undergoing apoptosis.

The PDCD5 gene is well conserved through evolution and display a high degree of homology in species ranging from yeast to mice. The level of PDCD5 protein expressed in cells undergoing apoptosis is significantly increased compared to normal cells. This has been shown in a study of Chen on TF-1 cells. The protein translocates rapidly from the cytoplasm to the nucleus of cells. This nuclear translocation of PDCD5 is a universal earlier event of the apoptotic process, and may be a novel early marker for apoptosis (Chen et al., 2001b).

B-cell lymphoma 2 protein

The bcl-2 gene encodes a Bcl-X_L -like protein with antiapoptotic effects. After an apoptotic stimulus, Bcl-X_L appears to localize primarily to the mitochondrial outer membrane where it might bind other apoptotic factors such as Bad and Bax or form an ion channel thought to maintain the integrity of the mitochondrial membrane and thus hindering the release of cytochrome-c (Chen, Gong et al, 2001)(Vogelstein et al.).

Bcl-2 can affect glycosylation pathways, and can function in two distinct ways to inhibit influenza virus induced cell death. It can block DNA fragmentation and apoptosis on an individual cell level, and reduce virus yield and the spread of infection to other cells. Apoptosis is an antiviral defense mechanism for the host, but it has been suggested that in the case of influenza virus, apoptosis may be important for optimal virus production (Olsen et al., 1996).

Myeloid leukemia differentiation protein -1

Myeloid leukemia differentiation protein-1 (Mcl-1) is an immune-relevant gene. Mcl-1 was originally found in inducible leukaemia cell lines and defined as an early induction gene. Mcl-1 belongs to the Bcl-2 family of genes (genes that were originally discovered from human B-cell lymphomas) that promote either cell survival or cell death (Adams and Cory, 1998).

Mcl-1 assists human neutrophil survival by rescuing them from cell death by apoptosis. Human Mcl-1 is induced by several cytokines (IL-1, IL-6, and IL-7) and other factors known to delay neutrophil apoptosis. This molecule is also essential in lymphoid development and later on in the maintenance of mature lymphocytes (Moulding et al., 1998).

Mcl-1 plays a critical role in preventing inappropriate activation of Bax and Bak, the key downstream mediators of cell death.

Mcl-1 has been reported to act at an apical step of the apoptotic pathway and must be eliminated or neutralized for apoptosis to proceed (Nijhawan et al., 2003). Neutralizing Mcl-1`s pro-survival activity is sufficient for apoptosis to proceed and that its degradation is not strictly required (van Delft and Huang, 2006).

3.6   Real-time reverse transcription polymerase chain reaction (RT-PCR)

3.6.1   Background and methodology

The reverse transcription polymerase chain reaction (RT-PCR) is the most sensitive method for detection of low-abundance mRNA, often obtained from limited tissue samples (Bustin, 2000). PCR is of particular value for expression studies in fish, because availability of antibodies is still limited (Jorgensen, S.M et al. 2005).

There are four commonly diagnostic tests used for detection of ISAV in Atlantic salmon. It is RT-PCR, indirect fluorescent antibody test (IFAT), virus culture and light microscopy. RT-PCR is the most sensitive method available for detecting and diagnosis of ISAV from Atlantic salmon (Snow et al., 2003a).

The real-time reverse transcription polymerase chain reaction (RT-PCR) uses fluorescent reporter molecules (like SYBR Green) to monitor the production of amplification products during each cycle of the PCR reaction. This combines the nucleic acid amplification and detection steps into one homogeneous assay. It has been shown that real-time PCR using SYBR Green is more sensitive than conventional RT-PCR for detection of nucleic acids (Bustin, 2000).

RT-PCR is an *in vitro* method for enzymatically amplifying defined sequences of RNA. As RNA cannot serve as a template for PCR, the first step in an RT-PCR assay is the reverse transcription of the RNA template into cDNA, followed by its exponential amplification in a PCR reaction. Usually this involves the use of dedicated RNA- and DNA-dependent DNA polymerase, either in separate or in single reactions. Alternatively, a single polymerase able to function both as an RNA and DNA-dependent DNA polymerase can be used in a "one-enzyme/one tube"-reaction. You can have a one step or a two step procedure;

in this study we have used a two step procedure. That means that the reverse transcription and the PCR-reaction are done in two steps.

First we started with normalized RNA-samples from cells which where converted to cDNA by using TaqMan DNA-polymerase in a Mastercycler. cDNA can be synthesised using random primers, oligo-dT, target gene-specific primers or a combination of oligo-dT and random primers (Bustin et al., 2005). The RT-PCR assay in this study was carried out using random primers. This approach primes the RT at multiple origins along every RNA template.

Oligo-dT is more specific than random priming, and is the best method to use when the aim is to obtain a faithful cDNA representation of the mRNA pool.

PCR is an elegant but simple technique for the in vitro amplification of target DNA utilizing DNA polymerase and two specific oligonucleotide or primer sequences flanking the region of interest. These primers hybridize to opposite strands of the DNA to serve as initiation points for the synthesis of new DNA strands. A thermostable DNA polymerase, such as Taq DNA Polymerase, catalyzes this synthesis. The principal function of DNA polymerase is to synthesize new strands of DNA in a 5-3 direction from a single stranded template. Most native DNA polymerase, however, are polyfunctional. In addition to their strand synthesis activity, many DNA polymerase are able to remove nucleotides sequentially from either end of the strand.

PCR is a cyclic process of double-strand separation of DNA by heat denaturation, specific hybridization or annealing of short oligonucleotide primers to single-stranded DNA. Each cycle doubles the region marked by the primer sequences. By sequential iteration of the process, PCR exponentially generates up to a billion of copies of the target within just a few hours (Fig 3.7). The specificity of PCR is highly dependent on the careful design of unique primers with respect to the genome under investigation and the nucleotide composition of the primer sequences.
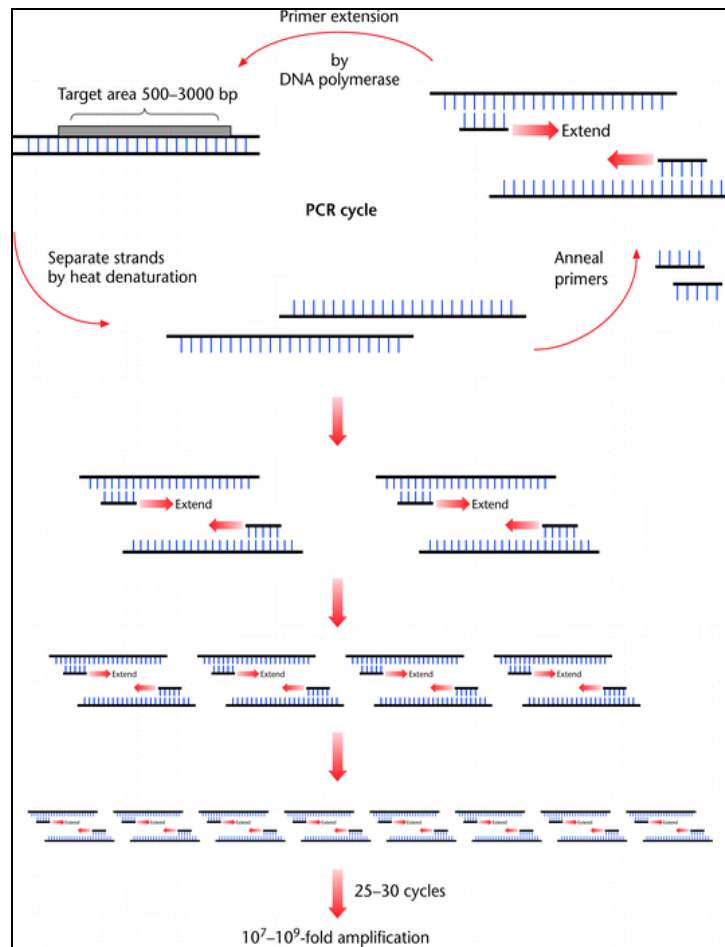
Fig 3.7: The PCR amplification cycle. Step 1: Denaturation. Heat (usually >90°C) separates double-stranded DNA into two single strands. This process takes place in a thermal cycler, an instrument that automatically controls the alternating cycles of heating and cooling required for PCR. **Step 2: Annealing.** The goal is not to replicate the entire strand of DNA but to replicate a target sequence that is unique to the organism. Primers define the ends of that target sequence. Primers are short, synthetic sequences of single-stranded DNA typically consisting of 20-30 bases. Annealing usually take place between 40 °C and 65 °C, depending on the length and sequence of the primers. This allows the primers to anneal specifically to the target sequence. **Step 3:** Once the primers anneal to the complementary DNA sequences, the temperature is raised to approximately 72 °C and a termostable polymerase (Taq DNA Polymerase) begins to synthesize new double-stranded DNA molecules which are identical to the original target DNA. It does this by facilitating the binding and joining of complementary nucleotides that are free in solution (dNTPs). Synthesis always begins at the 3` end of the primer and proceeds exclusively in the 5` to 3` direction. Thus, the new synthesis effectively extends the primers, creating a complementary, double-stranded molecule from a single-stranded template. At the end of the first PCR cycle, there are now two new DNA strands identical to the original target. (Figure from (Metzker, 2001)

The increase in fluorescence emission during the PCR reaction can be detected in real time by a modified thermocycler. The computer software constructs amplification plots using the fluorescence emission data that are collected during the PCR amplification. (Fig 3.8)
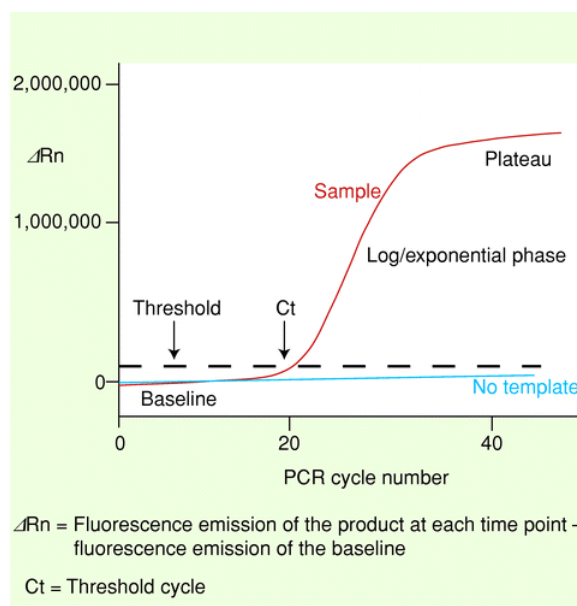
Fig 3.8: Amplification plot (from Arya, 2005)

The 7000 System SDS Software Version 1.2.3 sets baseline and cycle threshold automatically based on the data gathered from these phases.

The baseline is defined as the PCR cycles in which a reporter fluorescent signal is accumulating but is beneath the limits of detection of the instrument. By default, the computer software sets the baseline from cycles three to 15; however this needs to be changed automatically.

A computer software program calculates a deltaRn using the equation Rn=Rnf-Rnb, where Rnf is the fluorescence emission of the product at each time point and Rnb is the fluorescence emission of the baseline. The deltaRn values are plotted versus the cycle number. During the early cycles of PCR amplification, deltaRn values do not exceed the baseline.

An arbitrary threshold is chosen by the computers, based on the variability of the baseline. It is calculated as ten-times the standard deviation of the average signal of the baseline fluorescent signal between cycles three to 15. A fluorescent signal that is detected above the threshold is considered a real signal that can be used to define the threshold cycle (Clouthier et al.) for a sample (Arya et al., 2005).

Ct is defined as the fractional PCR cycle number at which the reporter fluorescence is greater than the minimal detection level. The Ct is a basic principle of real-time PCR as in an essential component in producing accurate and reproducible data (Higuchi et al., 1993). The presence of more template at the start of the reaction leads to a fewer number of cycles reaching the point at which the fluorescent signal is recorded as statistically significant above background (Gibson et al., 1996). This Ct value will always occur during the exponential phase of target amplification, which occurs during the early cycles of PCR. The real-time PCR are considered more accurate than the conventional PCR, because the amplification product is measured/detected during the log-linear phase of amplification when the conditions are optimum (Munir and Kibenge, 2004). The Ct value is inversely related to the amount of target templates in the samples (Niesters, 2001)

As reaction components become limiting, the rate of target amplification decreases until the PCR reaction is no longer generating template at an exponential rate (plateau phase) and there is little or no increase in PCR product (Arya et al., 2005).

3.6.2 Detection chemistries

There are two general chemistries available for amplicon detection. These include double-stranded DNA-intercalating agents (like SYBR Green) and fluorescent probes.

SYBR Green is a nonsequence-specific fluorogenic minor groove DNA-binding dye that intercalates into dsDNA (it does not bind to single-stranded DNA). SYBR Green exhibits little fluorescence when unbound in solution but emits a strong fluorescent signal upon binding to dsDNA (Fig 3.9) An increase in the fluorescence signal occurs during polymerization and this decreases when DNA is denatured. Fluorescent measurement is performed at the end of the elongation step of each PCR cycle to monitor the increasing amount of amplified DNA. The advantage for this procedure is that it can be used with any pair of primers for any target.
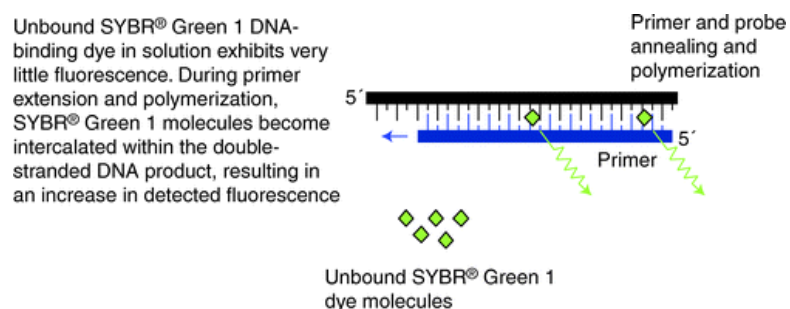
Fig 3.9: Double-stranded DNA-intercalating agents/DNA-binding dyes (e.g., SYBR® Green 1)
(figure from Arya, 2005)

However, as the presence of any dsDNA generates fluorescence, specificity of this assay is greatly decreased due to amplification of non-specific PCR products and primer-dimers (Ririe et al., 1997). Generating and comparing melting curves (plotting fluorescence as a function of temperature) is one method of increasing the specificity of the reaction (Ririe et al., 1997). A characteristic melting peak at the melting temperature (Papasavvas et al.) of the amplicon will distinguish it from amplification artifacts that melt at lower temperature at broader peaks (Arya et al., 2005).

### 3.6.3   Relative quantification

Relative quantification describes the changes in expression of the target gene relative to some reference group such as an untreated control or a sample at zero time in a time course study (Livak and Schmittgen, 2001). In real-time quantitative PCR-experiments errors will be introduced due to minor differences in efficiency of cDNA synthesis and PCR amplification. In order to minimize these errors and correct for sample-to-sample variation, a cellular RNA is simultaneously amplified with the target, which serves as an internal reference against which other RNA values can be normalized. The genes used for normalization is termed housekeeping genes. (Arya et al., 2005). In this study we have used 18S and EF1a as reference genes (Jorgensen et al., 2006).

To ensure that normalisation strategies are applied with proper statistical validity, mathematical models for real-time PCR quantification, such as REST 2005 (Pfaffl et al., 2002), have been developed and are used to analyze the real-time PCR results. The purpose of REST 2005 is to determine whether there is a significant difference between samples and controls, while taking into account issues of reaction efficiency and reference gene normalization.

3.7 Western blotting

Western blotting is a method by which proteins that have been separated and subsequently immobilized on the surface of a membrane are exposed and probed. Many variants of each step exist.

Blotting is the process of transferring physically separated analytes from a matrix to the surface of a membrane. The transfer step is preceded by one- or two- dimensional separation procedures and followed by a detection step that benefits from the increased accessibility of the transferred molecules on the blotting membrane surface. When antibodies are used as probes, the procedure is called immunoblotting. **Figure 3.10** shows the principle of electroimmunoblotting.
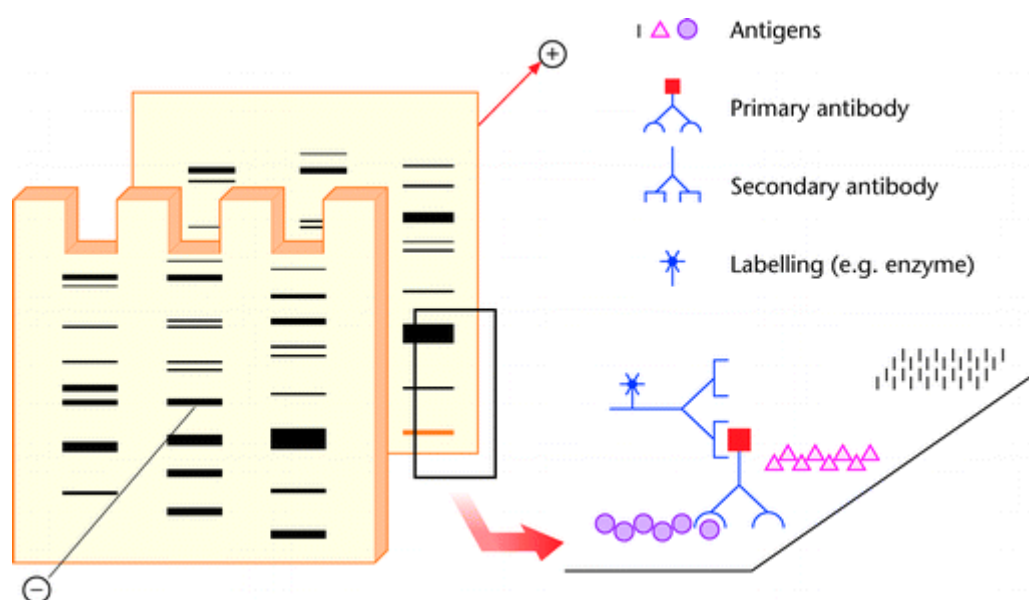
Figure 3.10: General principle of electroimmunoblotting. Protein bands in a gel are electrotransferred to a membrane and probed with primary antibodies. After washing, the bound primary antibodies are detected by enzyme-conjugated secondary antibodies that cause the development of precipitating colour after addition of the appropriate substrate. (Picture from (Fritschy, 2001))

The standard approach for protein immunoblotting involves a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) separation electrotransfer to nitrocellulose or polyvinylidene difluoride (PVDF) membranes.

3.7.1 Separation and transfer of the proteins

First the proteins are separated by gel electrophoresis, and then they are routinely blotted onto a membrane.

Transfer onto the membrane may be accomplished by molecular diffusion, by a buffer flow induced by suction or capillary action, or by using electrotransfer where charged molecules are transferred to the membrane by electrophoresis. Proteins from isoelectric focusing gels may be electrotransferred after addition of SDS (Stott, 1989)

Proteins separated in polyacrylamide gels are most often electroblotted. This usually works very well and takes place either in solution or in a semidry buffer system using wetted filter papers sandwiched between planar electrodes.

Diluted SDS-PAGE running buffer will work well as transfer buffer. The proteins are transferred toward the anode because of the anionic SDS and the slightly alkaline pH of the usual transfer buffers. Addition of methanol up to 20 % counteracts swelling of the gel and increases the binding of proteins to nitrocellulose. PVDF membranes (like Hybond-P membrane) exist in different variants, and they have to be wetted before use, e.g. in methanol.

After the membrane has been blotted it can be stained with different protein stains. This is done to make sure that the transfer of the proteins has worked well.
General protein staining with Ponceau S, Amido black, Coomassie blue or colloidal gold (in order of increasing sensitivity) is performed before the membrane is blocked with protein-solutions.

3.7.2 Blocking, probing and visualization of bound probes

After the proteins have been transferred to the membrane, the protein-binding sites on the membranes must be blocked to avoid non-specific binding in later steps. Much used are 1-5 % (w/v) solutions of proteins such as bovine serum albumin, non-fat dry milk, casein, ovalbumin, or gelatine that should not contain activities that will act on or inhibit the visualization of enzyme substrate. It is recommended to add 0.01-0.05 % Tween 20 in incubation and washing buffers to suppress unwanted background binding.

After the blocking step, the membrane is probed with antibodies. The primary antibody, which is unlabelled, is detected by a secondary labelled antibody that reacts with the molecules that remain bound after the first antibody incubation. Animal immune sera in appropriate dilutions can be used as probing reagents directly followed by visualization by a

broadly reacting secondary reagent, usually specific for γ-globulin class. Since antigen-antibody reactions are involved, the incubation and washing buffers are usually physiological with respect to pH and salt content.

The enzymes that are conjugated on the secondary reagents are most often alkaline phosphatase (AP) or horseradish peroxidase (HRP). In chemiluminescence methods enzymatic activity is converted to light, which is recorded on a photographic film. HRP systems commonly use Luminol/H2O2/*p*-iodophenol (Bjerrum, 2001).

3.8   Immunofluorescence microscopy

3.8.1   Background

Immunofluorescence is a biological assay combining the use of antibodies and fluorescent molecules for the detection of specific targets, like proteins, in cells and tissues. It is one of many techniques in biomedical research and diagnostics that makes use of the sensitivity and selectivity of fluorescence for analysing biological tissues. Immunofluorescence is based on the high selectivity and affinity of antibodies for their antigens as specific cellular constituents, notably proteins. Antibodies are made against purified antigen preparation, recombinant proteins, or synthetic peptides coupled to a carrier protein.

The sensitivity of immunofluorescence can be greatly improved by increasing the number of fluorescent molecules per antigen to be detected. This is usually done by indirect immunofluorescence. The primary antibody directed against the target structures or molecules are unlabelled. They are then bound by secondary antibodies raised against immunoglobulins of the host species used for the primary antibodies. The secondary antibodies are labelled either with fluorochromes or with other haptens, which serve as anchoring sites for enzymes or fluorescent molecules. Since the primary antibodies can bind more than one secondary antibody, the signal is amplified (Fritschy, 2001).

3.8.2 Fluorochromes

Different fluorochromes can be used in immunofluorescence. Carbocyanine (CY)-conjugated immunoreagents, which display a bright and stable fluorescence, are an excellent tool for indirect immunofluorescence labelling (Wessendorf and Brelje, 1992).

The strongly fluorescent Alexa dyes were developed for coupling to immunreagents (Panchuk-Voloshina et al., 1999). Different Alexa Fluor dyes is available, ranging from blue to red.

The fluorophores can be divided in four groups according to their colour-properties.

- Blue fluorescent dyes excited at wavelengths in the ultraviolet (UV) part of the spectrum (Alexa 350)

- Green fluorescent dyes excited by blue light (CY2, Alexa 488)

- Red fluorescent dyes excited by green light (CY3, Texas Red, Alexa 546)

- Dyes excited with far-red light, to be visualized with electronic detectors

3.8.3 Detection techniques for immunofluorescence

The confocal laser scanning microscope (CLSM) is an improved design of light microscope used for imaging fixed or living tissue that are usually labelled with one or more fluorescent probes. The advantage of the CLSM for imaging fluorescently labelled specimens is its ability to produce images, called optical sections, which are free from contaminating fluorescence from structures out of the focal plan of interest.

In the CLSM, a spot of light from a laser is focused into a specimen and scanned across it. Light that returns from the specimen is focused at a pinhole placed at a position that is confocal with the point in the specimen, and in front of a detector, usually a sensitive low-noise photomultiplier tube (PMT) that detects any light that passes through the pinhole.

The pinhole acts as a spatial filter, and prevents any light from above and below the focal plane of interest from reaching the PMT.

Most of the parameters for configuring the CLSM is controlled from the computer, for example laser brightness, filter combinations, the pinhole diameter and the sensitivity of the PMT.

Light from the laser source is directed, via a fibre-optic cable, into the scanning head, which is mounted on a conventional epifluorescence microscope stand. A combination of

optical filters similar to those used in a conventional epifluorescence microscope select the wavelength of light from the laser to excite the fluorochrome labelling the specimen.

Images are collected using digital methods, and usually with some form of signal averaging to remove electronic noise in the image caused by the PMT (Paddock, 2001).

3.9   The aim of the present study

The aim of this study was to monitor the expression of different stress-related genes that might be involved in the host defence-mechanisms against ISAV infection. Such analysis of genes induced by ISAV *in vitro,* may help us to better understand the pathogenesis of ISAV in Atlantic salmon. To test this, we used RT-PCR to measure the relative mRNA level of these genes after ISAV infection and poly I:C stimulation. ASK cells infected with ISAV was compared with cells stimulated with poly I:C (polyriboinosinic-polyribocytidylic acid). Poly I:C is widely used as synthetic dsRNA analog *in vivo* and *in vitro* and has also demonstrated to induce antiviral responses in Atlantic salmon. The candidate genes were partly identified by a recent microarray analysis of ISAV-induced genes and partly by literature surveys.

In recent years, two more antibodies directed against ISAV have been described, the 6AD/9Z antibody and the whole-virus antibody. Both antibodies are polyclonal but there were no published reports on their suitability in immunofluorescence microscopy. We therefore wanted to evaluate the applicability of these antibodies in immunofluorescence, and also test them in western blotting to evaluate if it can be used in diagnostics for detecting ISAV

4 MATERIALS

4.1 Reagents and chemicals

Applied Biosystems, Warrington, UK

SYBR®GREEN PCR Master Mix

Arcus, Oslo, Norway

Ethanol

BioRad

Laemmli sample buffer

Acrylamide

Bio Whittaker, Wokingham, UK

Foetal bovine serum (FBS, Australian origin)

Gentamicin (50 µg/µl)

Calbiochem, Merck, Germany

Tween 20

Cambrex Bio Sciences Rockland, ME, USA

FlashGel™ DNA Marker

FlashGel™ Loading Dye

Leibovitz (L-15) medium

Electro Microscopy Sciences, HA, UK

Para Formaldehyde 16 %

Eppendorf, Hamburg, Germany

Molecular Biology Graded Water

Gibco BRL, Uxbridge, UK

    2-mercaptoethanol (50 mM)

    L-glutamine (200 mM)

    Trypsin-EDTA

Invitrogen, Oregon, USA

    Prolong Gold with Dapi

    Alexa-phalloidin 568

Merck, Darmstadt, Germany

    NaCl

    HCl

    KH_2PO_4

    $Na_2HPO_4 \cdot 2H_2O$

    Methanol

Roche

    Complete tablets

Sigma-Aldrich

    TEMED

Non-fat dry skimmy-milk (Normilk, Levanger, Norway)

Triton-X-100

Ponceau S

4.2   Solutions

PBS, pH 7,4 (4 l)

32 g	NaCl
0,8 g	KCl
1,08 g	KH_2PO_4
7,12 g	$Na_2HPO_4 \cdot 2H_2O$
3950 ml	Deionized water

pH adjusted to 7,4 with HCL

Autoclaved, and stored at + 4 ˚C

Sucrose solution (20 %) 100 ml

20 g   Sucrose

ad 100 ml   Deionized water

TGS

100 ml 10 x TGS-SDS (BioRad):

       25 mM Tris

       192 mM glycine

       0,1 % (w/v) SDS

        pH 8,3

900 ml Deionized water

1 x TG (Blotting-buffer)

100 ml 10 x TG (BioRad)

       25 mM Tris

       192 mM glycine

       pH 8,3

200 ml Methanol

700 ml Distilled water

10 % SDS-Page gel(0,75 mm-2 geler)

Resolving gel:

3,0 ml   MQ

1,8 ml   Lower buffer

2,4 ml   Acryl amide ?

3,6 μl   TEMED

36 μl   APS

Stacking gel:

2,4 ml	MQ
1,0 ml	Upper buffer
0,6 ml	Acryl amide
4,0 µl	TEMED
20 µl	APS

4.3 Kits

RNeasy® Mini Kit (Qiagen, MD, USA)

RNase free DNase set (Qiagen, MD, USA)

TaqMan® Reverse Transcription Reagents (Applied Biosystems, NJ, USA)

FlashGel™ System Kit (Cambrex Bio Science Rockland, ME, USA)

4.4 Antibodies

α-Tubulin (12G10)- (Frankel and Nelsen, Department of Biological Sciences, University of Iowa, USA)

α-ISAV-HE, 6AD/9Z-(kindly provided by B.Krossoy, Department of Fisheries and Marine Biology, University of Bergen, Norway), Polyclonal

α-ISAV Whole-virus-(kindly provided by B.Krossoy, Department of Fisheries and Marine Biology, University of Bergen, Norway), Polyclonal

α-ISAV-HE,Falk-(kindly provided by K.Falk, National Veterinary Institute, Oslo, Norway) Monoclonal

α-Galectin-9-(Santa Cruz Biotechnology, Inc, California, USA)

Goat anti-mouse HRP,Goat anti-rabbit HRP,CY2-Rabbit anti-mouse,CY2-Mouse anti-rabbit (Jackson, Immuno Research, Suffolk UK)

Alexa Fluor 488 Rabbit anti-goat (Invitrogen, Oregon, USA)

4.5   Primers

Real-time PCR primers used in this study

Genes	Direction	Sequence	E	Amplicon size	Acc.no
18S	F R	TGTCCGCTAGAGGTGAAATT GCAAATGCTTTCGCTTTCG	1,00	61	AJ427629
EF1α	F R	CACCACCGGCCATCTGATCTACAA TCAGCAGCCTCCTTCTCGAACTTC	0,97	77	AF321836
IFNα	F R	CCTGCCATGAAATCCTACTCGGA TTTCCTGATGAGCTCCCATGC	1,00	107	AY216594
Mx	F R	TGATCGATAAAGTGACTGCATTCA TGAGACGAACTCCGCTTTTTCA	1,00	80	SSU6677
sshsp90	F R	GAATGACAAGGCGGTGAAGG AGAGCGGTCTCGAAAAGCAG	0,97	51	AF135117
sshsp70	F R	ACCCAGCTATGTGGCCTTCA GCTGCGTCTCCGATGAGTCT	0,98	52	AJ632154
ssil1β	F R	GGAGAGGTTAAAGGGTGGCG TCCTTGAACTCGGTTCCCAT	1,06	51	AY617117
sstnfα	F R	CGGGTTCAAGCTACAAGGGA AAGAGCCCAGTGTGTGGGAT	1,21	51	DQ787158
sscox2	F R	GCGGTTTATTCCGGATCCTC GCGAAGAAGGCGAACATGAG	1,20	51	AY848944
ssfip2	F R	GCCTTGTAGAGGCCCTCTCC CCACCTTCATGCTGAGCTCC	1,01	51	DQ496225
ssogf1	F R	GCAAAGCAGAGGCTGGTCAC GCCGTAGAAGTCCAGCATGAG	1,02	51	AM259613
ssisg15-2	F R	TGTTAGGTGTCAATGGGAGCAA TGTGTCTGGCCCTTTTCGTT	1,03	151	AY926456

Table 4.1: Primers for RT-PCR used in the present study. The primers were already designed and tested. E= primer efficiencies

Real-time PCR primers used in this study

Genes	Direction	Sequence	E	Amplicon SIZE	Acc.no
cIAP-1(2)b	F R	AGAACAGCCCGTTCCTCATG CCGAGCGAAGGTGGAGATAC	0,98	101	TC43301
Pdcd5b	F R	AACCGACGAAAGGTGATGGA CCTGAATCCCTCCCTAAGCC	0,98	101	TC29121
Caspase3a	F R	GGAATGAGCTTTCGCAATGG ACAGTCTGGTCATTGGCAACC	0,91	101	DQ008069
p53	F R	AAAACCCCCGGCAATAACAA CTAGGACCTGAAAGCAGCAGC	1,00	151	BG934348
bcl21a	F R	TTGTCAGTTGGTGCTGGAGG CCAAATTGCTTCTGCCGTTC	0,93	101	TC29759
Gal_9_1	F R	TTAACCTGCGTTTCAACTCGG TGGACCCCACTGTTCCTTCA	1,16	101	AF483533
Nfκb-1-inh	F R	CCTTGTTGATCTCGGCGCT GCAGCACCAAGTCCAGGTTC	0,98	101	CA388461
p62	F R	TGATGCCTCATCTGCTGCC CGATACCCAGTGGGCTAAGC	1,04	102	CA375694
trans1	F R	ACCTGCTTACCATTTCCCCC CAAATCACAGGCCTTGGCTT	1,01	100	TC25489
Mcl1-1	F R	TTGACTTGGATGACCGATGC TGGCCACAGAATTGATGACG	1,12	51	AM259614

Table 4.2: Primers for RT-PCR used in the present study. The primers were already designed and tested. E = primer efficiencies

5   METHODS

5.1   Cell-cultures

ASK cells (Atlantic salmon kidney cells), kindly provided by B.Krossoy (department of Fisheries and Marine Biology, University of Bergen, Norway) were cultured in Leibovitz L-15 medium (Cambrex Bio Sciences, Verviers, Belgium) supplemented with 50 µg/ µl gentamicin, 4mM L-glutamine, 40 µM β-mercaptoethanol and 10 % foetal bovine serum (L-15 complete) The cells were split 1:3 every week and maintained at 20°C.

5.2   Virus production

Inoculation of ISAV 4 (strain Glesvaer 2/90), with a titer of 1x $10^{6.8}$ $TCID_{50}$/ml was performed on ASK-cells grown in 500 cm^2 cell culture flasks. The cell culture medium were removed and saved for later use. The cells were washed three times with phosphate buffered saline (PBS), pH 7.4, and 20 ml ISAV inoculate, diluted 1:50 in serum-free medium were added to the flasks. The virus was allowed to adsorb for 4 hours at 15 °C. After 4 hours, 100 ml L-15 complete medium were added. Infection was allowed to proceed at 15 °C for 20 days to ensure enough virus replication.

Cell culture supernatant from ISAV-infected ASK-cells was cleared from cell debris by low speed centrifugation at 4000 x g for 20 minutes at 4 °C (Beckman Avanti J-25 centrifuge, with JA-10 rotor). The supernatant that contained the virus was decanted to a sterile tube.

To further separate virus from remaining serum proteins, 32 ml of the cell culture supernatant was poured in six polyallomer tubes (Beckman). Then 4 ml 20% sucrose solution was added gently to the bottom of the tubes using a syringe, ensuring that the two layers does not mix together. The tubes containing the supernatant and sucrose solution were then centrifuged at 104000 x g for 2 hours at 4 °C (Sorvall Discovery 100 centrifuge with Surespin 630 rotor). The sucrose step was repeated so all the virus was pelleted and filtered through the sucrose solution.

The six tubes containing the virus pellets were then washed two times with PBS to remove remaining medium and sucrose solution. 2 ml of PBS was applied to one tube and a sonicator-rod (Vibra Cell, Sonic & Materials inc., Danbury, CT, USA) was used three times for 2 seconds to resuspend the pelleted virus in the PBS solution. The tubes were kept on ice

to keep from heating during sonication. The PBS solution containing the ISA virus was then moved from tube to tube, with disruption of the pelleted virus in every tube. The final 2 ml PBS solution containing all the virus from all six tubes were either diluted in medium (inoculate) or stored as a pellet at -80 °C for later use.

5.3   Poly I:C stimulation

ASK cells were seeded in 25 cm^2 cell-culture flasks and grown for at least 24 hours before stimulation. Poly I:C (Sigma Aldrich, St.Louis, USA) was dissolved in 0, 9 % NaCl (5 mg/ml). 50 µl poly I:C (50 µl ml^{-1}) was added directly to the cells. Cells were harvested at day 1 and 3 post-stimulation.

5.4   Virus infection of ASK cells

ASK-cells were seeded in 25 cm^2 tissue-culture flasks. Each flask was seeded with 4 x 10^5 cells in 5 ml L-15 complete medium. (Concentration of 1.5 x 10^4 cells/cm^2). The cells were then grown for at least 48 hours at 20 °C before infection with ISAV.

After 48 hours, the medium was removed from the flasks and stored for later use. The cells were washed three times with PBS before 1 ml ISAV4 inoculate, diluted in serum-free medium was added. The ASK-cells were infected with a MOI of 1. The control-flasks were mock-infected with 1 ml serum-free medium instead of ISAV-inoculate. The flasks were incubated on a rock tray "The Belly Button" (Stovall Life Science Greenboro, NC, USA) for 4 hours at 15 °C.

After 4 hours of inoculation the stored medium was diluted 50:50 with serum-free medium, and 5 ml was added to each flask. The flasks were stored at 15 °C until collection. Cells were collected at 1, 3 and 5 days post infection (p.i.).

Before adding the lysis-buffer RLT, the cells were washed three times with PBS to remove the medium so that it does not inhibit the lysis-buffer. After the washing procedure, the cells were lysed with RLT-buffer directly in the cell-flask and scraped off with a rubber policeman, and homogenized according to the instructions of the manufacturer (Rneasy® Mini Kit). It was then stored at -80 °C until later use.

5.5   RNA-extraction

Total RNA was isolated from lysed cells using RNeasy® Mini Kit with on-column RNase free DNase set (Qiagen, MD, USA), according to manufacturer's instructions. The DNase set were used to remove small amounts of DNA that can interfere with the results in the RT-PCR. Total RNA was eluted in a final volume of 30 µl RNase free water (Eppendorf, Hamburg, Germany). The purity and quantity of the RNA was found by measuring the optical density (OD) at 260 nm/280 nm on a Beckman DU®530 Spectrophotometer by using 3 µl RNA diluted in 97 µl RNase-free water. The RNA-samples were stored at -80 °C for later use.

5.6   cDNA synthesis

All RNA-samples were reverse transcribed to cDNA using TaqMan® Reverse Transcription reagents according to the protocol from the manufacturer (Applied Biosystem, CA, USA) in a total volume of 100 µl. A total of 1 µg of RNA was used from each sample.

The reagents used for each sample:

10 µl	10x TaqMan RT buffer
22 µl	25 mM Magnesium Chloride (cofactor)
20 µl	deoxyNTPs Mixture
5 µl	Random Hexamers
2 µl	RNase inhibitor
2.5 µl	MultiScribe Reverse Transcription
1 µg	RNA
38.5-amount of RNA solution used	RNase-free water

Total volume of 100 µl

The cDNA synthesis was performed in an Eppendorf MasterCycler, at 25 °C for 10 minutes (annealing), 48 °C for 30 minutes (polymerisation), 95 °C for 5 minutes (denaturation) and 4 °C for maximum 60 minutes.

5.7   Real-Time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Real-time PCR was performed in 96-well optical plates on an ABI PRISM 7000 Sequence Detection system (Applied Biosystems). Each well contained a final volume of 25 μl, containing 12,5 μl SYBR® Green Master Mix, 3 μl reverse primer, 3 μl forward primer and 1,5 μl Molecular Biology Graded Water (Eppendorf, Hamburg, Germany) and 5 μl of cDNA diluted 1:10, except for the cDNA used for the 18S-primers, which were diluted 1:1000. For each primer-set we used a control. Instead of 5 μl cDNA, 5 μl of Molecular Biology Graded Water was added to the well. For each cDNA-sample we used two parallels. This was done to make sure that the two parallels showed the same result, and that the wells were not contaminated during mixing of the reagents.

The housekeeping-genes EF1a and 18S were used as reference genes, since they have been shown to have the best stability in infected cells (Jorgensen et al., 2006).

The PCR reactions were performed at 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute.

All sample setups were standardized and wells centrifuged for 2 minutes at 100 x g at 4˚C prior to analysis.

5.8   Real-time PCR data analysis

Data handling was done according to the ABI Prism 7000 Sequence Detection System User Manual and baseline and cycle threshold (Clouthier et al.) were set automatically for each PCR-reaction. High C_t-values (over 35) were discarded. Relative mRNA transcription levels of the genes were calculated relative to levels in control cells (mock-infected), and normalized to the expression of both 18S and EF1-α.

To calculate the relative expression of the target genes, C_t-values of the target genes and reference genes were analyzed using the Relative Expression Software Tool (REST©) (Pfaffl et al., 2002). Both the ISAV-infection trial and the poly I:C-stimulation were repeated three times.

The results from the analyze were transferred to SigmaPlot 3.0, and relative expression graphs were made, showing the change in transcription level for both ISAV infected cells and poly I:C stimulated cells over time.

5.9   Primer amplification products test

The real-time PCR products from control day 3 were tested by agarose gel electrophoresis. This was done to make sure that we did not have two PCR-products with different amplicon size in the wells. Agarose gel electrophoresis was done with FlashGel™ System Kit (Cambrex Bio Science Rockland, ME, USA).

Each sample well was loaded with 7 µl deionized water. The first well was added 5 µl FlashGel™ DNA Marker. The other wells were added a mix of 1 µl of DNA solutions from the samples, and 4 µl FlashGel™ Loading Dye. The samples were run at 150 volts, until the desired separation was obtained.

The fragments on the FlashGel™ Cassette were then illuminated by UV-light using Chemi Genius^2 (Bio Imaging System). The results were viewed and pictures were taken.

5.10   Western blotting

ASK cells were infected with ISAV (strain 2/90 Glesvaer), according to the virus-infection protocol described earlier, and samples from day 1, 3 and 5 post-infection were collected, as well as a control-sample(mock-infected) from day 3. The samples were washed two times with PBS. On forehand, a stock solution of 1 tablet Complete dissolved in 2 ml PBS was made. This Complete-solution contains phosphatase- and proteolytic inhibitors. A mixture of sample buffer Laemmli Sample (960 µl) with β-mercaptoethanol, and Complete-solution (40 µl) was added directly to the cells, and the cells were scraped off with a rubber policeman. The solution was homogenised with a syringe (21 gauge), and heated for 5 minutes at 95°C. The proteins were on reduced form.

Before the samples were separated, the protein content was measured using the BCA Protein kit (Pierce, Rockford, IL, USA) according to the manufacturer's protocol. Based on a standard curve, the protein contents in each sample are calculated.

The proteins were separated using a 10 % polyacrylamide SDS-Page gel. 20 µl of each sample was added to the wells. 5 µl Precision Plus Dual Colour Standard (BioRad) was used as a standard. A 10 x TGS running-buffer was used, and the samples were run at 100 volts for 1.5 hours.

The proteins were blotted to a Hybond™-P membrane (Amersham Biosciences UK Ltd) overnight at 4 °C at 30 V, by using electroblotting. The membrane was stained with Ponceau S before it was blocked.

The protein blot was then treated with 5 ml blocking solution (PBS containing 0.1 % Tween 20 % and 5 % non-fat dry milk) for 1 hour at room temperature. After 1 hour, the primary antibody was added directly to the blocking solution in different dilution, depending on which primary antibody to use. The membrane was incubated with the primary-antibody for 1 hour at room temperature.

The membrane was washed 3 times with PBS containing 0.1 % Tween 20 for 15 minutes. After the membrane was washed it was incubated with a secondary horseradish peroxidise (HRP)-conjugated antibody diluted in blocking solution for 1 hour at room temperature. The membrane was then washed 3 times in PBS containing 0.1 % Tween 20 for 15 minutes.

Antibody concentration:

Tubulin

Primary:	Tubulin (12G10)	1:500
Secondary:	Goat-Anti Mouse HRP	1:5000

6AD/9Z

Primary:	6AD/9Z	1:1000
Secondary	Goat-Anti Rabbit HRP	1:10000

Whole-virus

Primary:	Whole-virus	1:500
Secondary:	Goat-Anti Rabbit HRP	1:5000

After the membrane was incubated with secondary antibody, and washed three times, the membrane was placed on a plastic film. ECL plus (Amersham Biosciences UK) was used as a reagent to detect bound HRP-conjugate. 25 µl Lumigen Solution B was mixed with 975 µl Lumigen Solution A and added to the membrane. It was left there for 5 minutes, and the liquid was removed. Chemiluminescence was detected using ECL plus detection reagent. A developer machine from Kodak was used to develop the membranes on photo paper.

5.11 Immunofluorescence-microscopy

ASK-cells were seeded in 10 cm^2 six-well tissue-culture plates with 3 coverslips per well. The cells were counted, and 1.5 x 10^5 cells were seeded in each well with 3 ml L-15 complete medium. The tissue-culture plates were incubated at 20 °C for at least 48 hours.

After 48 hours the medium was removed and stored for later use, and the cells were washed three times with PBS. The ISAV inoculate (2/9 Glesvear) was diluted in serum-free L-15 medium, and 600 µl of the ISAV-inoculate was added to each well. The virus was allowed to adsorb at 15 °C for 4 hours and were incubated on a rock tray "The Belly Button" (Stovall Life Science Greensboro, NC, USA). The mock-infected wells were added 600 µl serum-free medium.

After 4 hours, 3 ml of a 50:50 mix of the saved medium and L-15 complete medium was added to each well. The wells were sealed with PCR Adhesive Sealing Sheets (Eppendorf, Hamburg, Germany), and stored at 15 °C for later collection.

Samples were taken out on day 1, 3 and 5 post-infection. The medium was taken off, and the cells were washed three times with PBS.

The cells were fixated with 1 ml 4 % PFA (16 % Formaldehyde diluted in PBS) per well for 20 minutes. After 20 minutes, the cells were washed two times with PBS.

The coverslips were incubated on a drop (40-50 µl) of Triton-X-100 (permeabilization of the plasmamembrane) on parafilm for 10 minutes. This will permeabilize the cells. Then the coverslips were blocked on a drop of 5 % Bovine Serum Albumin (BSA) (Sigma Aldrich, USA) on parafilm for 1 hour at room temperature. The coverslips were then incubated with a drop of primary antibody diluted in 2.5 % BSA at 4 °C overnight.

The coverslips were washed three times with PBS for 15 minutes, and then they were incubated with a secondary antibody diluted in 2.5 % BSA for 1 hour at room temperature. Then the washing procedure with PBS was repeated. The coverslips were mounted on object slips with Prolong Gold with Dapi (Invitrogen, Oregon, USA). It was left to dry until it was viewed on a confocal-microscope (Nikon).

The coverslips incubated with Falk, 6AD/9Z and whole-virus was also treated with phalloidin 568 diluted 1:40 in PBS for 15 minutes.

Antibody used in this study:

Falk

- The monoclonal 3H6F8 antibody was obtained by immunizing mice with purified virus particles

Primary:	Falk (3H6F8)	1:200
Secondary:	CY2-Rabbit anti-mouse[*]	1:800

6AD/9Z

- The 6AD/9Z antiserum was obtained by immunization of rabbits with HE peptides MGDSRSDQSRVNPQSC and CPKMVKDFDQTSLGNT coupled to keyhole limpet haemocyanin

Primary:	6AD/9Z	1:50
		1:100
		1:200
Secondary:	CY2-Mouse anti-rabbit[*]	1:800

Whole-virus

- The whole virus antiserum was obtained by immunizing rabbits with purified virus particles.

Primary:	Whole-virus	1:50
		1:100
		1:200
Secondary:	CY2-Mouse anti-rabbit[*]	1:800

Galectin-9

-is an affinity purified goat polyclonal antibody raised against a peptide mapping near the C-terminus of galectin-9 of human origin.

Primary:	Galectin-9	1:50
		1:100
		1:200
Secondary:	Alexa Fluor 488-Rabbit anti-goat	1:800

The secondary antibody (marked with [*)] should be spun down in a Microfuge-Biofuge Fresco centrifuge at 13000 rpm for 15 minutes at 4 °C. This is done because the secondary antibody is stored at -20 °C dissolved 1:1 with glycerol, and aggregates can easily be formed.
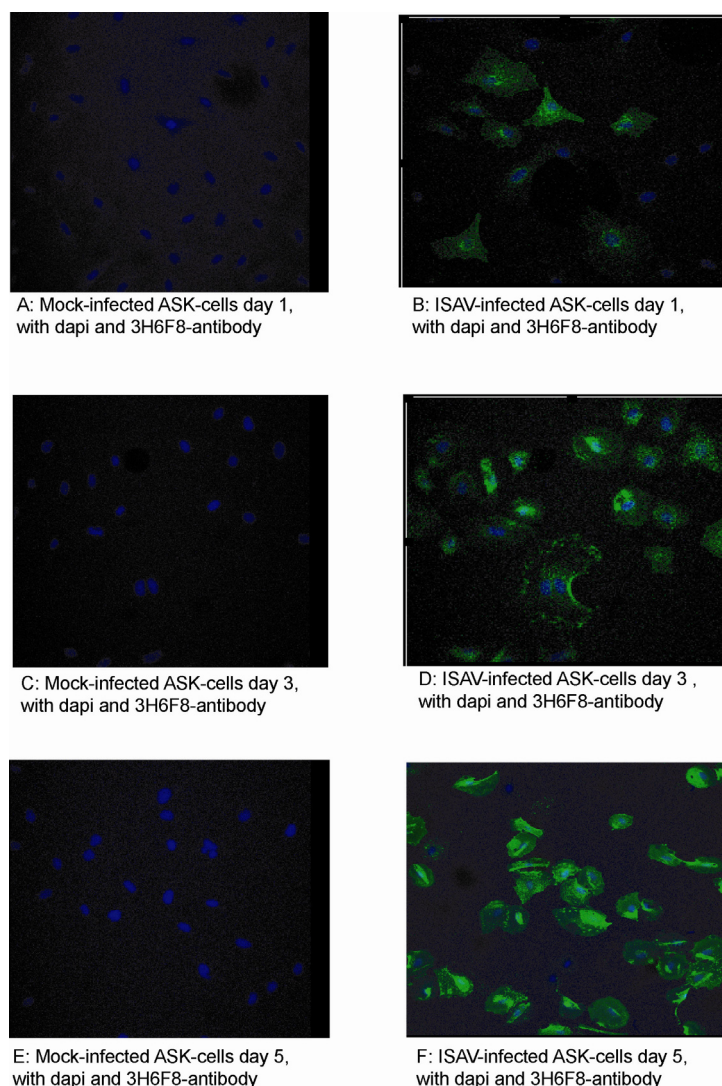
6   RESULTS

6.1   Viral replication/infection

To monitor the ISAV infection we used confocal immunofluorescence-microscopy. ASK cells infected with ISAV from day 1, 3 and 5 post infection , including mock-infected cells from day 5 were fixed to coverslips, and incubated with a primary antibody, 3H6F8 (Falk-antibody) as described in 6.11, and viewed in a confocal-microscope.

The antibody 6AD/9Z and the whole-virus antibody were also tested in immuofluorescence and in western blot to evaluate their specificity.

6.1.1   Detection of viral replication/infection using Falk-antibody (3H6F8)

As mentioned earlier, ISAV has been shown to possess both hemagglutinating and receptor-destroying activity. The Falk-antibody (3H6F8) is a primary antibody directed against hemagglutinin-esterase on the surface of ISAV (Fig 6.1).

A: Mock-infected ASK-cells day 1, with dapi and 3H6F8-antibody

B: ISAV-infected ASK-cells day 1, with dapi and 3H6F8-antibody

C: Mock-infected ASK-cells day 3, with dapi and 3H6F8-antibody

D: ISAV-infected ASK-cells day 3, with dapi and 3H6F8-antibody

E: Mock-infected ASK-cells day 5, with dapi and 3H6F8-antibody

F: ISAV-infected ASK-cells day 5, with dapi and 3H6F8-antibody

Fig 6.1: Immunofluorescence. Immunofluorescence staining with 3H6F8 (Falk)-antibody of ASK cells infected with ISAV. The samples are from day 1, 3 and 5 post infection. The cells were also counterstained with DAPI.

No fluorescence was observed in mock-infected cells compared to the ISAV-infected ASK cells. This confirms that the 3H6F8-antibody is bound specifically to virus infected ASK cells.

At day 1 post infection, around 80 % of the ASK cells were infected with ISAV. In a later state of the infection, at day 3 and 5, all of the cells were infected with ISAV. This shows that the ISAV replicates and spreads through the cell culture. Figure 6.2 shows close-up pictures of the ISAV-infected ASK cells at day 1, 3 and 5 post infection.
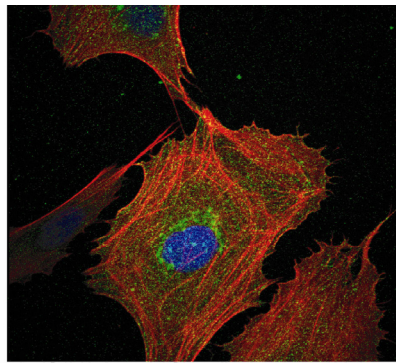
A : ISAV-infected ASK-cells day 1 p.i. (60x)
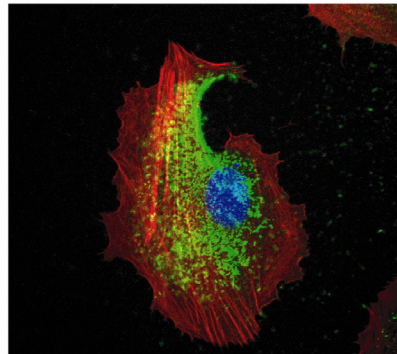
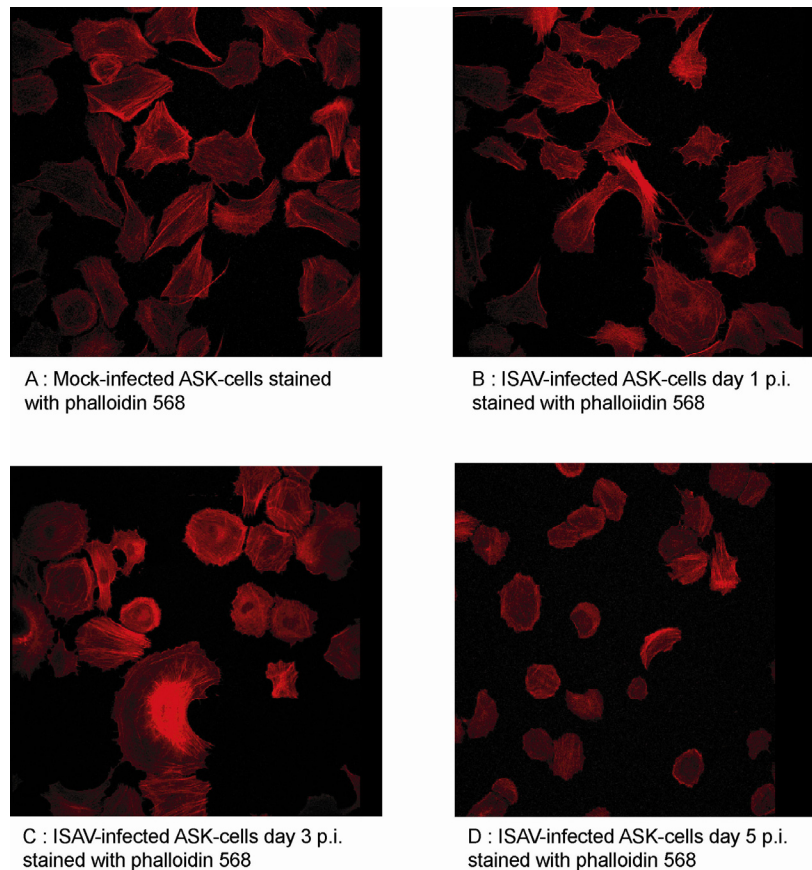B : ISAV-infected ASK-cells day 3 p.i. (60x)
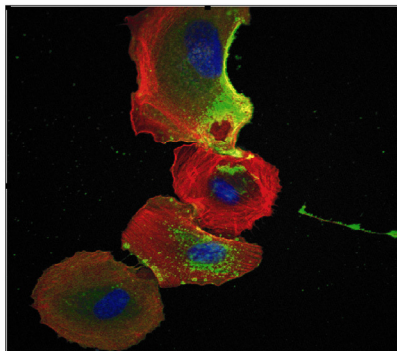
C : ISAV-infected ASK-cells day 5 p.i. (60x)

Figure 6.2: Immunofluorescence. Immunfluorescence staining of ASK cells infected with ISAV incubated with 3H6F8 (Falk)-antibody (Chipuk et al.). The cell nucleus is stained with dapi (blue), and the aktin-filament is stained with phalloidin 568 (red). The pictures were taken with the 60X objective.

Figure 6.2 show that we have an ISAV infection in the cells already from day 1 p.i. The ISAV infection is evident in day 3 and 5 p.i.

It was also observed that the ISA-virus is caused a cytopatic effect (CPE) in the ASK cells. In figure 6.3 we see ASK cells that either are mock-infected (A) or infected with ISAV (B,C and D). The actin cytoskeleton in the cells was stained with Alexa-phalloidin 568, as shown in figure 6.3.

A : Mock-infected ASK-cells stained with phalloidin 568

B : ISAV-infected ASK-cells day 1 p.i. stained with phalloiidin 568

C : ISAV-infected ASK-cells day 3 p.i. stained with phalloidin 568

D : ISAV-infected ASK-cells day 5 p.i. stained with phalloidin 568

Figure 6.3: Cytopathic effect of ISAV. Confocal micrographs of ASK cells, mock-infected (A) or infected with ISAV (B,C and D) was stained with Alexa-phalloidin 568.
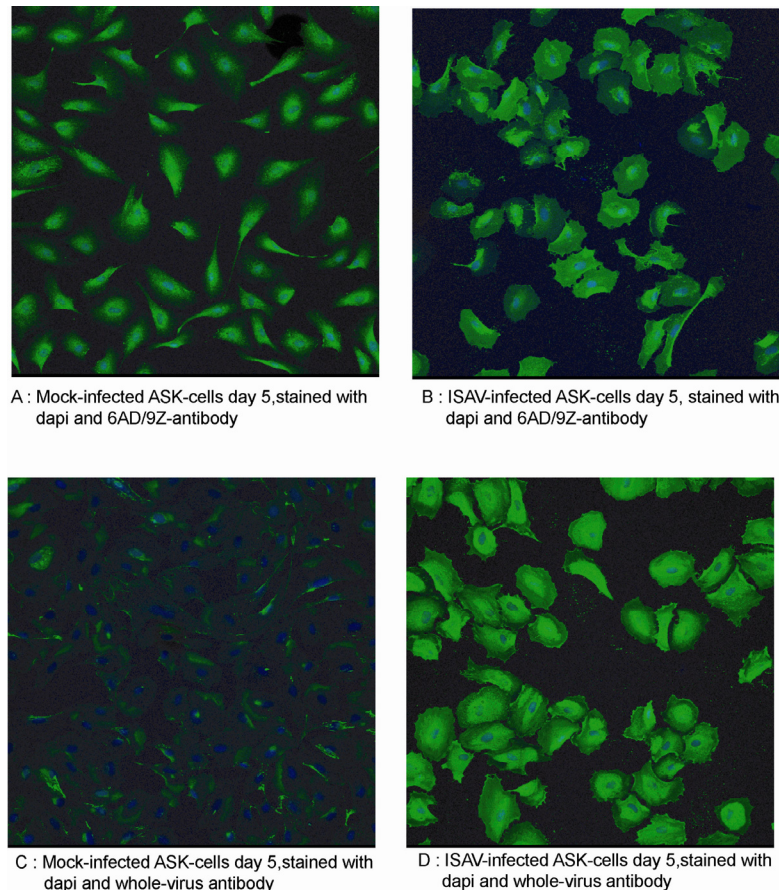
Figure 6.3 shows that the actin-filament is changed from day 1 till 5 post infection (B,C and D), compared to the control-sample (A). ISAV induced a cytophatic effect (CPE) already after 3 days. The cells loosened from the plastic cell culture plates, and we could see a small change in morphology of some cells from a flattened, to a rounder shape. The mock-infected cells did not loose from the plates in the same extend. At day 5 p.i. (D) we observed marked changes in the morphology of the cells.

In the poly I:C stimulation trials, we could see an effect of the treatment already at day 1. At day 3 we could clearly see that the cells were affected, and loosened from the plastic plates (not shown).

6.1.2 Detection of viral replication/infection using 6AD/9Z and whole-virus antibody

Two alternative antibodies, 6AD/9Z and whole-virus, that is either directed to a peptide sequence in the hemagglutinin-esterase on the surface of ISAV or to the whole-virus, respectively were alto tested for their cross reactivity with infected cells. These antibodies has previously only been tested in western/immunoblot (Krossoy et al., 2001), so we wanted to look at the possibilities of using these antibodies in immunofluorescence to detect the ISAV-infection.

This was tested at day 5 post infection to ensure many virus positive cells. Antibody concentration and the blocking-solutions were varied to find the optimal conditions. Figure 7.4 shows ASK-cells that were incubated with either 6AD/9Z or whole-virus antibody in a dilution of 1:200, using BSA as a blocking solution.

A : Mock-infected ASK-cells day 5, stained with dapi and 6AD/9Z-antibody

B : ISAV-infected ASK-cells day 5, stained with dapi and 6AD/9Z-antibody

C : Mock-infected ASK-cells day 5, stained with dapi and whole-virus antibody

D : ISAV-infected ASK-cells day 5, stained with dapi and whole-virus antibody

Figure 6.4: Immunofluorescence. Immunofluorescence staining with 6AD/9Z (A and B) or whole-virus antibody(C and D) of ASK cells that either are mock-infected or infected with ISAV. 6AD/9Z is diluted 1:200, and whole-virus antibody is diluted 1:200.

In figure 6.4 we can see that the fluorescence is shown in both mock-infected and ISAV-infected ASK cells. This indicates that both 6AD/9Z and whole-virus antibody also binds cellular proteins, and they were not specific to the proteins in ISAV. We tried to change the primary antibody concentration and the blocking solution to see if we could get better and clearer results, but without any improvement (not shown).

6.1.3 Detection of virus-infection using Western blotting (immunoblotting)

AS the 6AD/9Z and whole-virus antibodies gave non-specific background in immunofluorescence, we wanted to try if we could get better results in western blotting.
ASK cells were infected with ISAV, as described in chapter 5.2 and taken out at day 1, 3 and 5 post infection. Mock-infected cells were used as a control-sample. The cells were lyzed in sample buffer containing β-mercaptoethanol and heated. The proteins were separated on a SDS-Page gel, and probed with 6AD/9Z and whole-virus primary antibody in concentrations shown in section 6.10. It was then developed with a HRP-conjugated secondary antibody. Chemiluminescence was detected using ECL plus detection reagent.

Tubulin-antibody (12G10) was used as a positive control on the same ASK cells samples. Tubulin has a molecular weight of 47 kDa. Detection of tubulin would serve as a loading control of the samples.
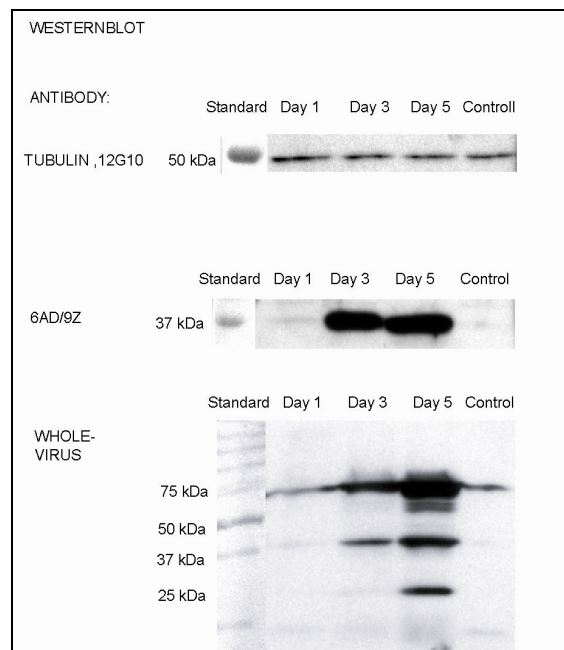
Fig 6.5 Westernblots Immunoblots showing ASK cells infected with ISAV, day 1, 3 and 5 post infection, and mock-infected ASK cells as a control, incubated with either anti-Tubulin (12G10), 6AD/9Z or whole-virus primary antibody.

In the westernblots we can see that bands were detected around 50 kDa when the samples were probed with a α-Tubulin antibody. The bands were detected in all the infected samples, as well as the control sample.

Further, bands are detected at approximately 40 kDa when the membrane was probed with 6AD/9Z-antibody. The band is only detected in ISAV-infected samples from day 3 and day 5 post infection. A weak band was also detected at 40 kDa at day 1 post infection.

In western blots using whole-virus antibody as a probe, we observed 3 (4) different bands at day 5 p.i. with molecular weights around 75 kDa, 37 kDa and 25 kDa, and also a narrow band around 50 kDa. At day 3 we only see proteins at 75 kDa and 37 kDa. And in day 1 and control-sample we can only see proteins at approximately 75 kDa. The signal in the control lane is most probably a result leakage from the neighbouring lane.

6.2 Effect of ISAV infection and poly I:C treatment on gene expression in ASK cells

ASK cells were either infected with ISAV as described in section 5.4, or stimulated with poly I:C as described in section 5.3. Samples from the ISAV infection trials were taken out at day 1, 3 and 5 post infection. In the poly I:C trials, the samples were taken out at day 1 and 3 post stimulation.

All expression data were normalized to two housekeeping genes, 18S and EF1-α These genes have been validated by Jørgensen et al, for use as reference genes in quantitative real-time PCRstudies in Atlantic salmon (Jorgensen et al., 2006). The figures displayed below, show the mean relative expression and the standard deviation for each gene tested in both poly I:C-stimulated and ISAV-infected cells. The relative expression of the different genes in both poly I:C-stimulation and ISAV-infection were compared to see if there was any variation.
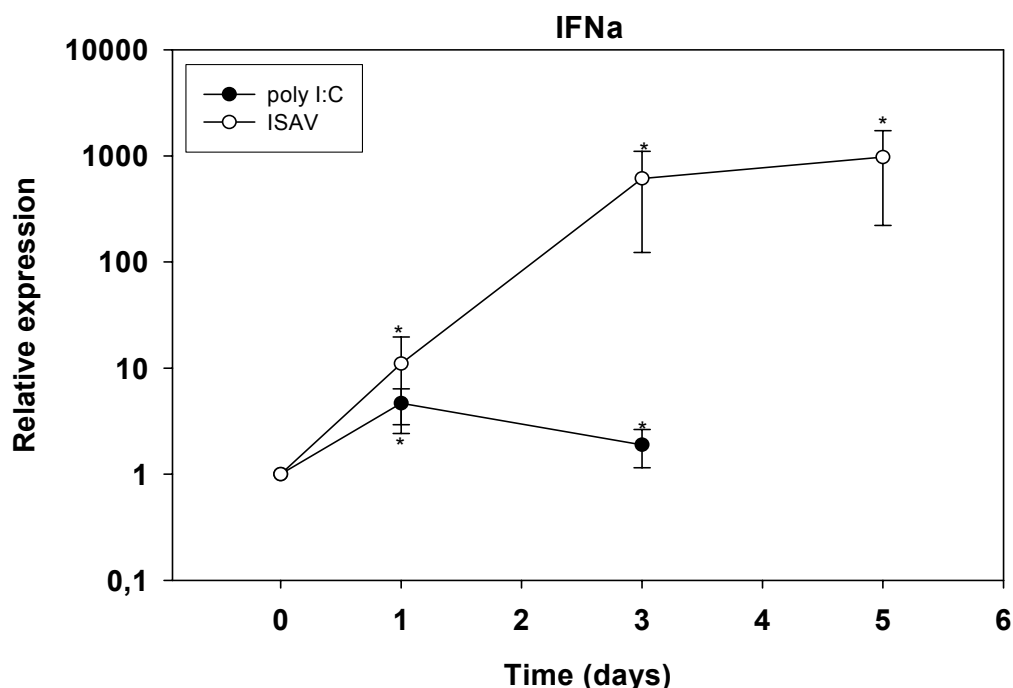
Fig 6.6: Relative expression of IFNα in ASK cells. IFNα is a component of the antiviral immune-response. IFNα is expressed early after a viral-infection Expression is calculated in REST relative to mock-infected cells and normalized to housekeeping gene 18S and EF1-α. (* = statistic significant result)
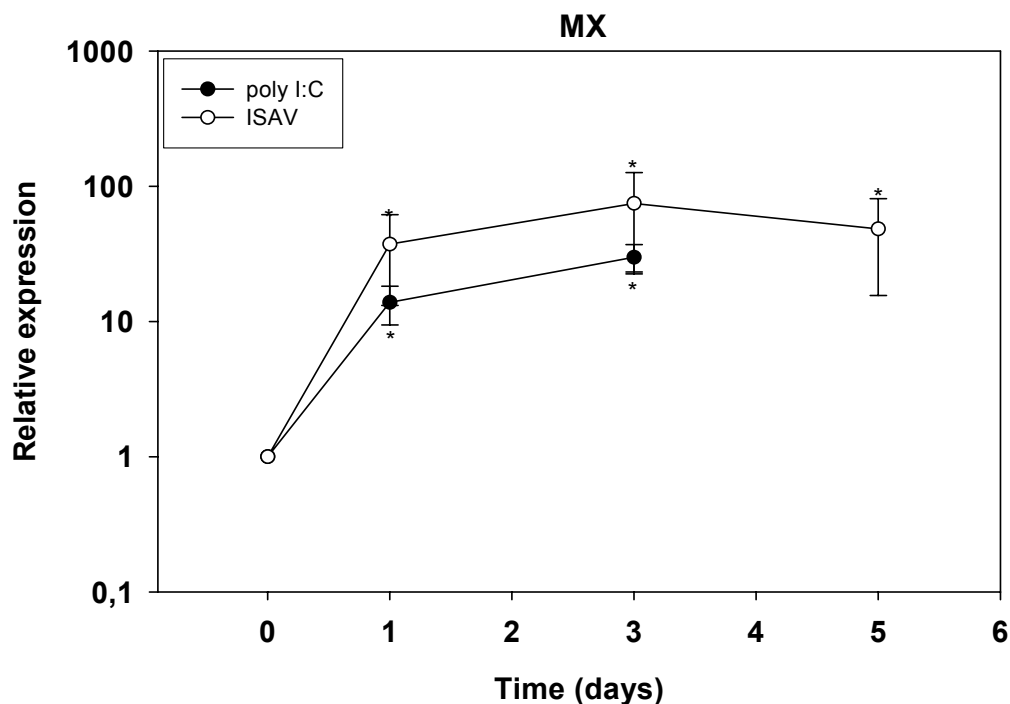
Fig 6.7: Relative expression of Mx in ASK cells. Mx proteins are key components of the antiviral state induced by interferons. Expression is calculated in REST relative to mock-infected cells and normalized to 18S and EF1-α (* = statistic significant result)
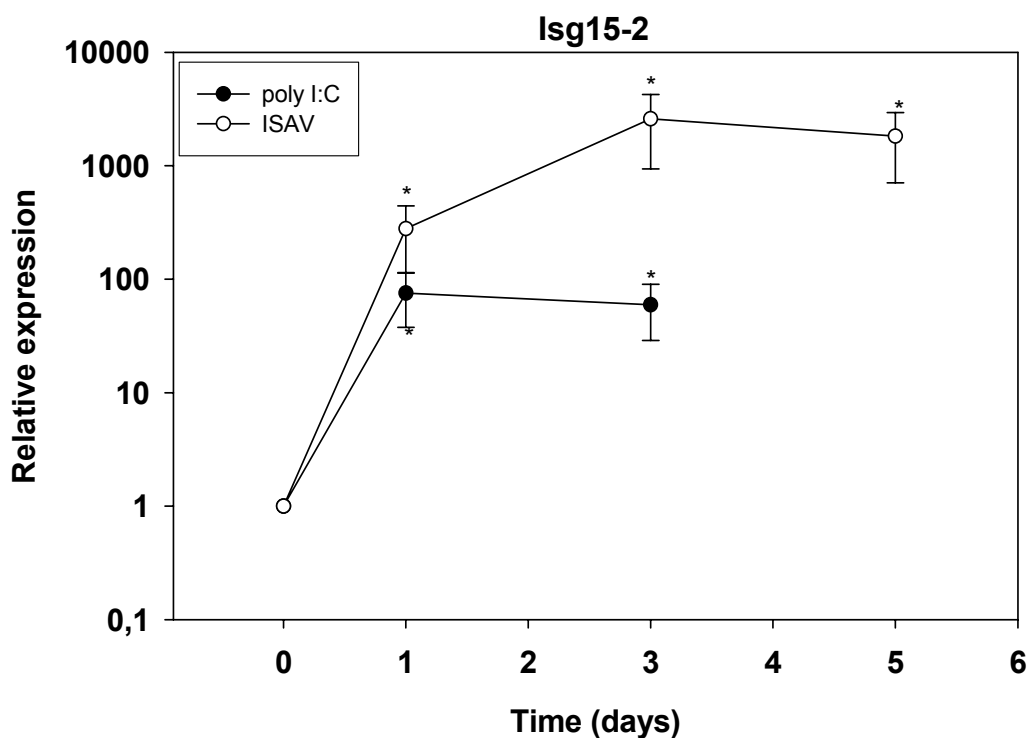
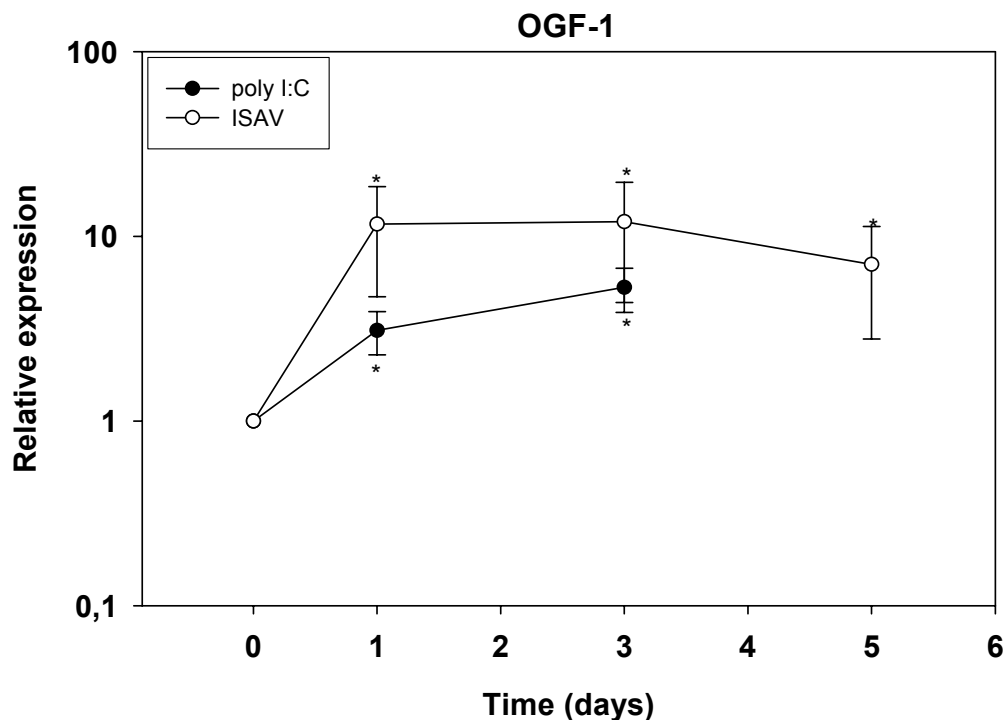**Fig 6.8: Relative expression of ISG15 in ASK cells.** ISG15 is an important host IFN-induced antiviral protein that functions *in vivo* against several important human pathogens. Expression is calculated in REST relative to mock-infected cells and normalized to 18S and EF1-α (* = statistic significant result)

Relative expression of interferon-α and interferon-induced genes:

As shown in figure 6.6, IFN-α was up-regulated about a 10-fold already 1 day post infection in ISAV infected cells. In poly I:C stimulated cells IFN-α was up regulated 5 fold compared to control. At day 3 post infection the difference in the induction of IFN-α expression in ISAV infected and poly I:C stimulated cells becomes more pronounced. The expression of IFN-α in ISAV-infected cells are increased to a 1000-fold up-regulation. In the poly I:C stimulated cells we can see a decrease to a 2 fold up-regulation. The IFN-α expression was up-regulated to about a 1000-fold at day 5 post infection in ISAV infected cells. The poly I:C treatment was only tested at day 1 and 3 post stimulation.

As a result of the up-regulation of IFN-α, we observed an induction of the IFN-inducible antiviral Mx gene. As seen in figure 6.7, Mx is up-regulated in both ISAV infected and poly I:C stimulated cells at day 1 and 3 post infection. At day 1 post infection we observed a 10-fold up-regulation in poly I:C stimulated cells and approximately a 50-fold up-regulation in ISAV infected cells compared to mock-infected samples. The relative expression of Mx steadily increased to day 3 post infection in both experiments.

In figure 6.8 we see a strong up-regulation of ISG15 (Interferon stimulated gene 15) in both poly I:C-stimulated and ISAV-infected cells. Already at day 1 post infection there was a 400 fold up-regulation in ISAV and a 90 fold up-regulation in poly I:C. This clearly indicates that it is an important gene in the interferon-response. In the ISAV-infected cells we can see that the relative expression of ISG15 increases steadily to day 3 p.i, further we can see a decrease in the expression at day 5 p.i. In poly I:C-stimulated cells a decrease in relative expression of ISG15 already at day 3 p.i to an 80 fold up-regulation. All these changes were statistically significant according to the statistical program, REST. This results strongly indicates that both IFN-α, Mx and ISG15 is up-regulated in cells that are either ISAV infected or poly I:C stimulated.
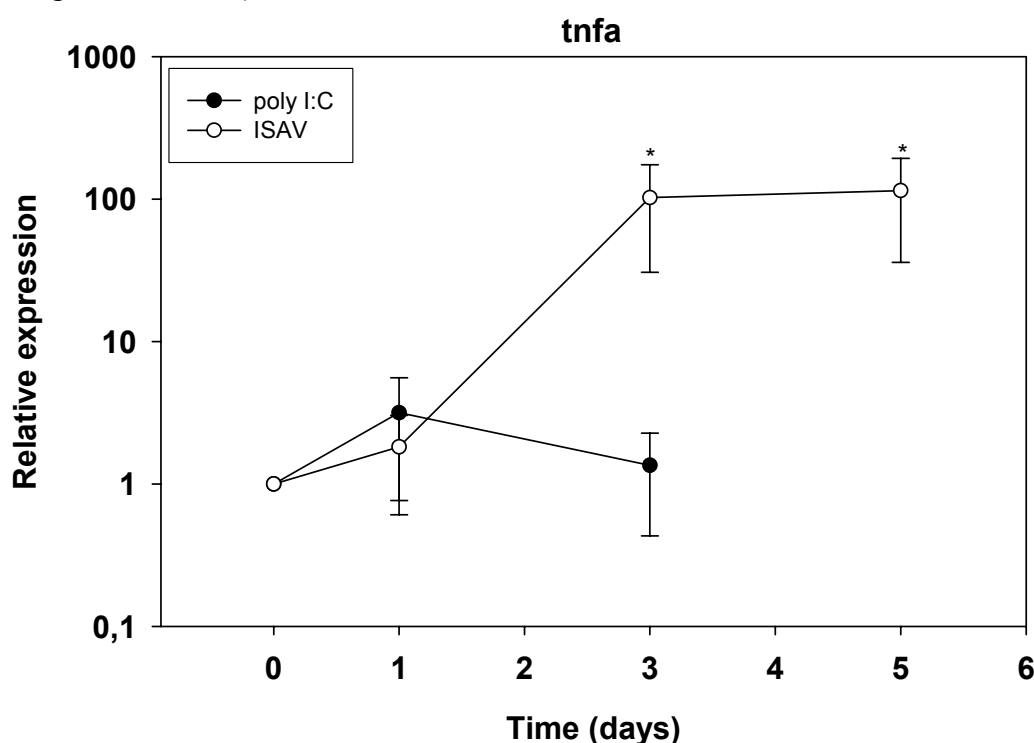
Fig 6.9: Relative expression of Ogfr1 in ASK cells. Opioid growth factor receptor (Ogfr) is a negative regulator of cell-proliferation. Expression is calculated in REST relative to mock-infected cells and normalized to 18S and EF1-α. (* = statistic significant result)

Relative expression of Ogf-1:

As shown in figure 6.9, opioid growth factor receptor was up-regulated in both ISAV infected cells and poly I:C stimulated cells compared to mock-infected cells. But as can be seen from the figure, there was a stronger up-regulation in ISAV infected cells. In ISAV infected cells the expression of Ogfr is up-regulated 10 fold at day 1 and day 3 but decreased to 8-fold up-regulation at day 5 p.i. In poly I:C a 4 fold at and 6 fold up regulation was observed at day 1 at day 3, respectively. These changes were also statistically significant, and indicates that Ogfr expression is up-regulated in both ISAV and poly I:C stimulated ASK cells.
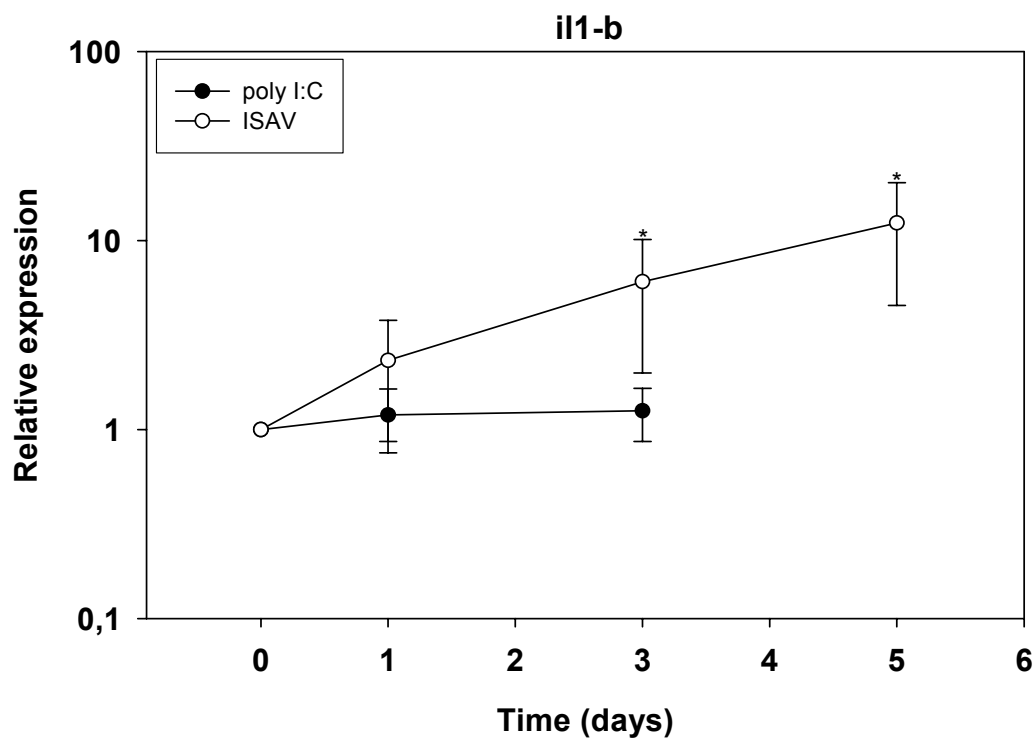
Fig 6.10: Relative expression of il1-β in ASK cells. Interleukin-b (Il1β) is a cytokine which is a mediator in the inflammation-response. Expression is calculated in REST relative to mock-infected cells and normalized to 18S and EF1-α. (* = statistic significant result)

**Fig 6.11: Relative expression of tnfα in ASK cells.** Tumor necrosis factor-α (tnfα) is a cytokine that is produced in many different cell-types in an inflammation-response, infection and injuries. It can enhance the anti-viral defence mechanism. Expression is calculated in REST relative to mock-infected cells and normalized to 18s and EF1-α (* = statistic significant result)

<u>Cytokine expression:</u>

The cytokine interleukin-1 was up-regulated in ISAV infected cells. The relative expression of il-1 increased steadily from day 1 to day 5 p.i, till it reaches a 10 fold up-regulation at day 5 (fig 6.10) In poly I:C stimulated cells no change in gene-expression of interleukin-1 compared to control was observed.

The cytokine tumor necrosis factor-α was up-regulated in ISAV infected cells and to a smaller extend in poly I:C-stimulated cells (fig 6.11). A small up-regulation of the gene-expression at day 1 increased to a 100 fold up-regulation at day 3 and 5 p.i., for ISAV infected cells. In poly I:C stimulated cells, no significant changes were observed.
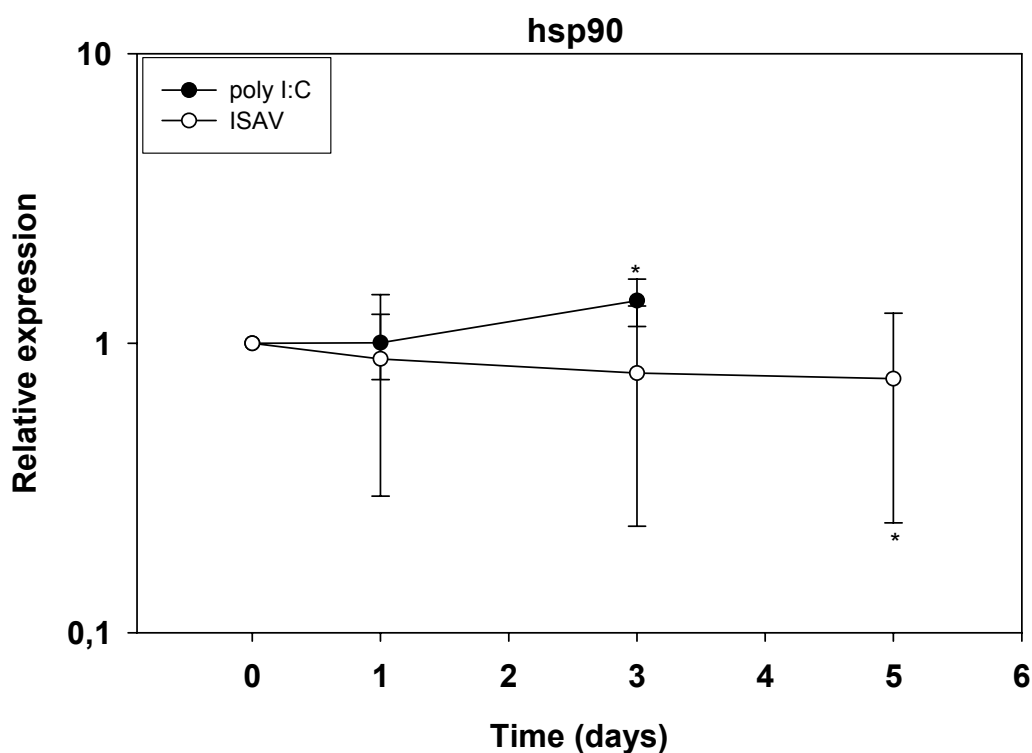
Fig 6.12: Relative expression of hsp90. Heat shock protein 90 (hsp90) is synthesized after stress, and is involved in cell-cycle control. Expression is calculated in REST relative to mock-infected cells and normalized to 18S and EF1-α. (* = statistic significant result)
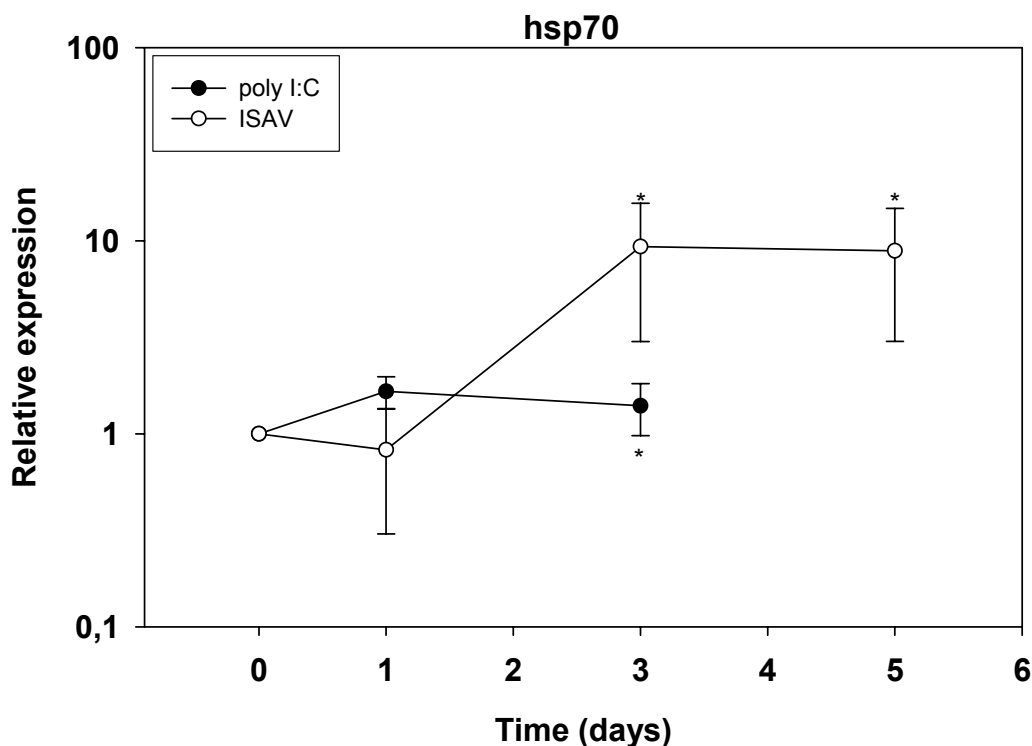
Fig 6.13: Relative expression of hsp70. Heat shock protein 70 (hsp70) is a chaperone that is induced by different stress-responses, like virus-infection. Hsp 70 is highly expressed in cells that experience stress-responses. Expression is calculated in REST relative to mock-infected cells and normalized to 18S and EF1-α. (* = statistic significant results)

Relative expression of the heat shock proteins, hsp90 and hsp70:

As can be seen in figure 6.12, neither ISAV infection or poly I:C stimulation had any strong effects on hsp90 expression in these cells.

Hsp70 was up-regulated in both ISAV infected cells and poly I:C stimulated cells at day 3 p.i. In poly I:C stimulated cells a 2 fold up-regulation of hsp70 compared to 10-fold in ISAV infected cells.
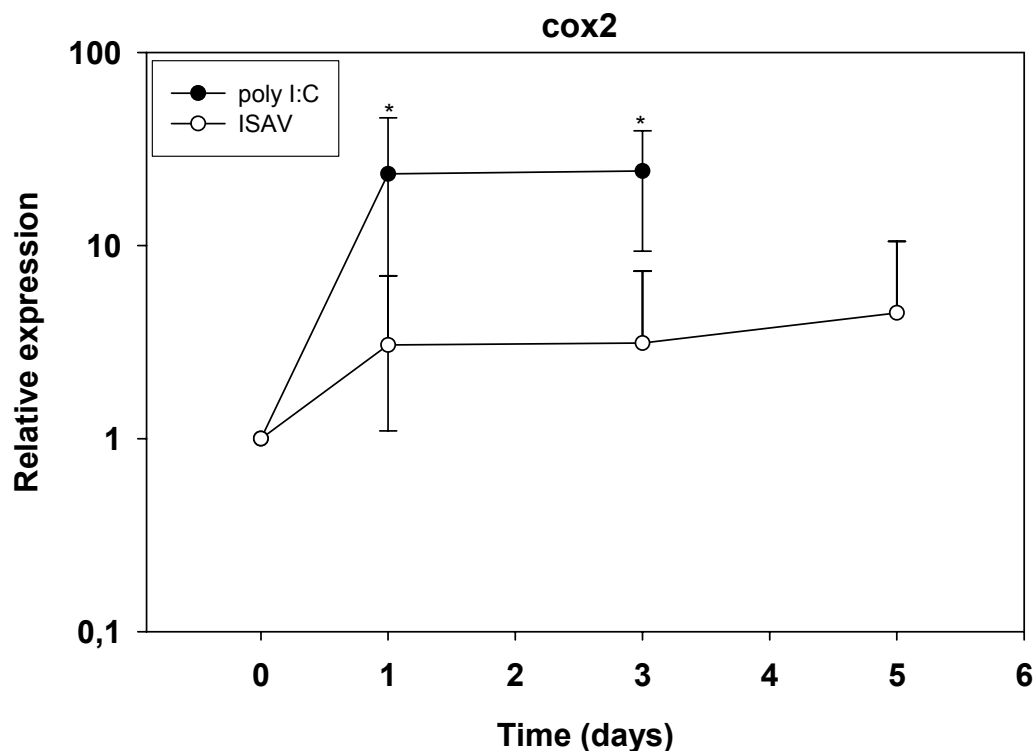
Fig 6.14: Relative expression of cox-2 in ASK cells. Cyclooxygenase-2 is important for the synthesis of prostaglandins that is important in inflammation. Expression is calculated in REST relative to mock-infected cells and normalized to 18S and EF1-α. (* = statistic significant result) The whole standard-deviation bar is not shown due to that the standard deviation came under the x-axis (negative log-value is not allowed)
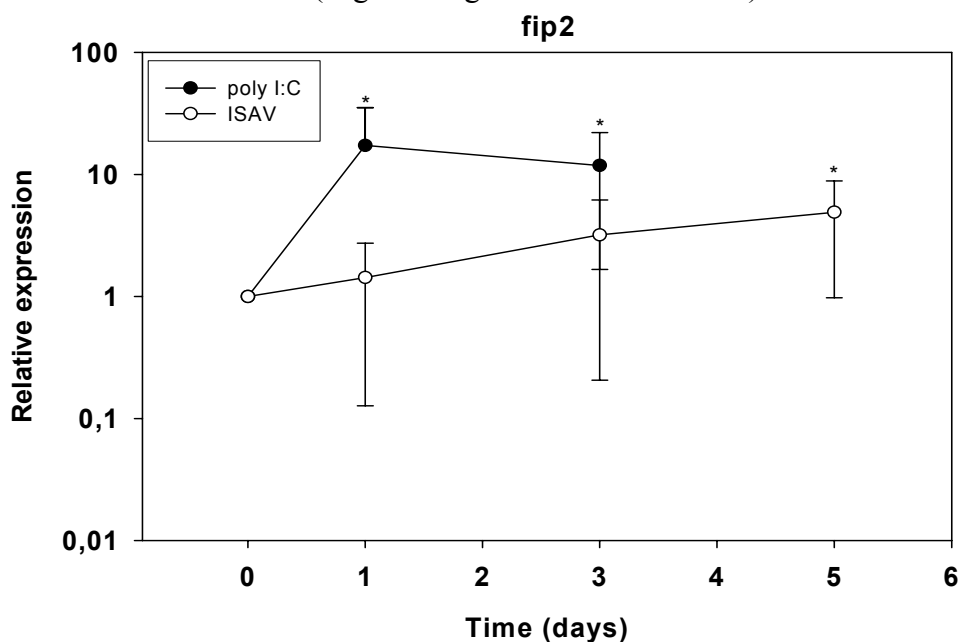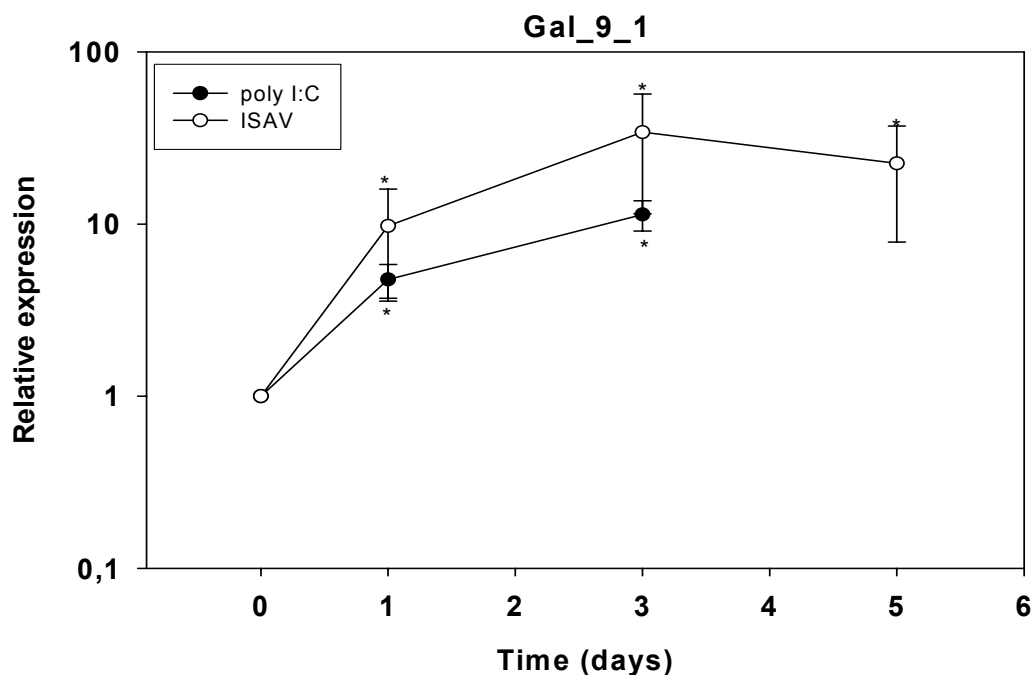

Fig 6.15: Relative expression of fip2 in ASK cells. Fourteen Kilodalton Interacting Protein 2 (Fip2) is a protein that potentially is involved in tnfα/NF-κB signaling. Fip2 is also involved in cellular remodulation. Expression is calculated in REST relative to mock-infected cells and normalized to 18S and EF1-α. (* = statistic significant result)
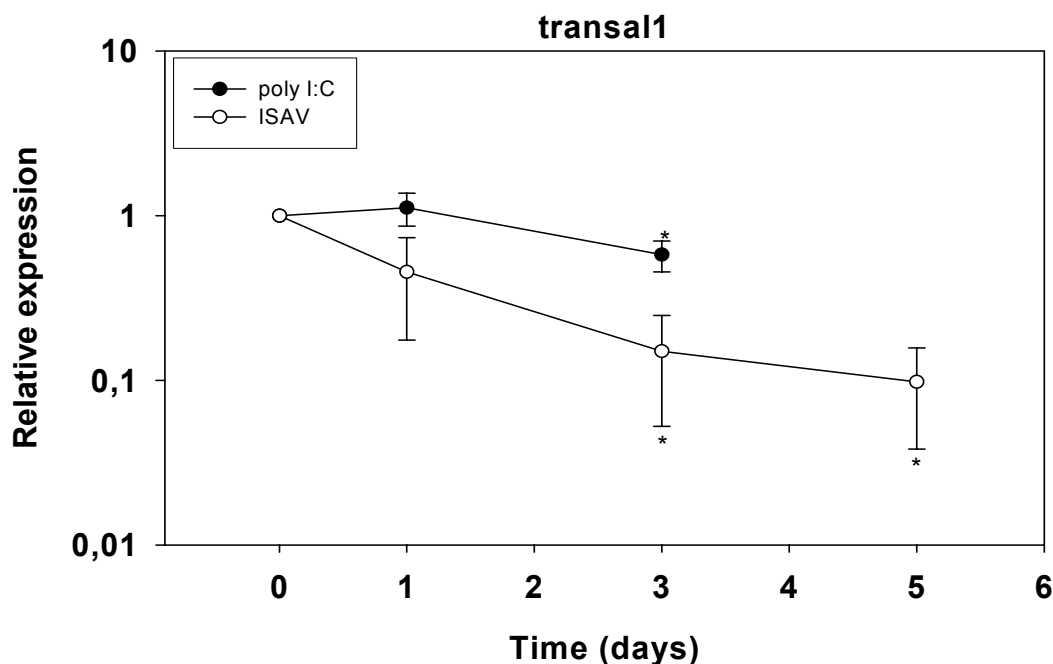
Relative expression of cox-2 and fip-2:

Figure 6.14 shows that cox-2 was up-regulated 40-fold in poly I:C stimulated cells. At both day 1 and day 3 p.i. In ISAV infected cells no significant changes were observed.

In figure 6.15 shows that there was an up-regulation of fip-2 both in poly I:C stimulated and ISAV infected ASK-cells, but with more rapid kinetics in poly I:C stimulated ASK cells compared to infected. Already after day 1 p.i we can see an 20 fold up-regulation in poly I:C, whereas it took 5 days before ISAV infected cells displayed a statistically significant up-regulation.

Fig 6.16: Relative expression of Galectin-9 in ASK cells. Galectins are a family of soluble β-galactoside-binding animal lectins that modulate cell-to-cell adhesion and cell-to-extracellular matrix interactions and play a role in tumor progression, pre-mRNA splicing, and apoptosis.Galectin-9 can induce chemotaxis of eosinophiles, and apoptosis of T-cells. Expression is calculated in REST relative to mock-infected cells and normalized to 18S and EF1-α. (* = statistic significant result)

**Fig 6.17: Relative expression of transaldolase 1 in ASK-cells.**
Transaldolase is a key-enzyme in pentose-phophate pathway. A reduced
transaldolase expression would lead to inhibition of apoptosis. Expression
is calculated in REST relative to mock-infected cells and normalized to
18S and EF1-a.(* = statiscic significant result)

Relative expression of galectin-9 and transaldolase-1:

Galectin-9 (Gal-9) is highly up-regulated in both poly I:C stimulated and ISAV
infected ASK cells as can be seen in figure 6.16. Gal-9 was more induced in ISAV than in
poly I:C stimulated cells, but the expression level shows the same tendency in both
experiments. There was a 10 fold up-regulation already at day 1 in ISAV infected cells, where
poly I:C stimulated cells shows a 7 fold up-regulation. The up-regulation increased steadily
from day 1 till day 3 p.i. in both experiments, until it decreased in day 5 in ISAV infected
cells. These results shows a up-regulation in both poly I:C stimulated and ISAV infected cells.

The relative expression of transaldolase is down-regulated in both poly I:C stimulated
and ISA infected cells. But there is a stronger down-regulation of transaldolase-expression in
ISAV infected cells. This result strongly indicates that transaldolase is down-regulated after
ISAV infection, and as well in poly I:C stimulated cells at day 3.
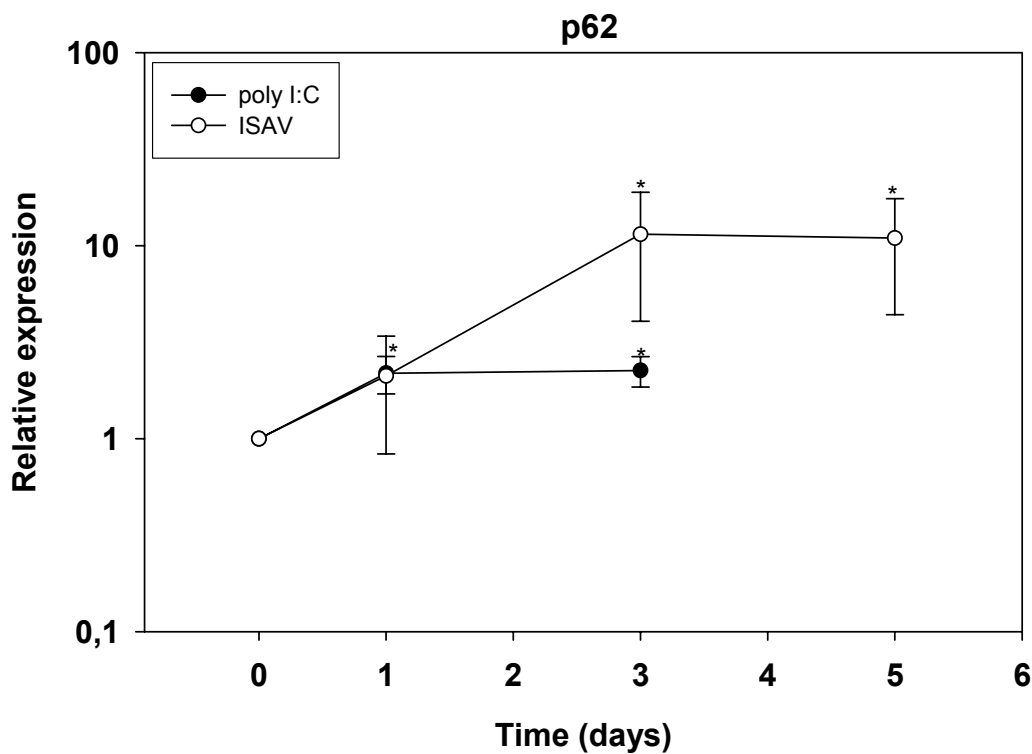
Fig 6.18: Relative expression of p62. p62 is an adaptor-protein that bind ubiquitin and regulates the signal cascade through ubiquination.Expression is calculated in REST and normalized to18S and EF1-a.
(* = statistic significant result)
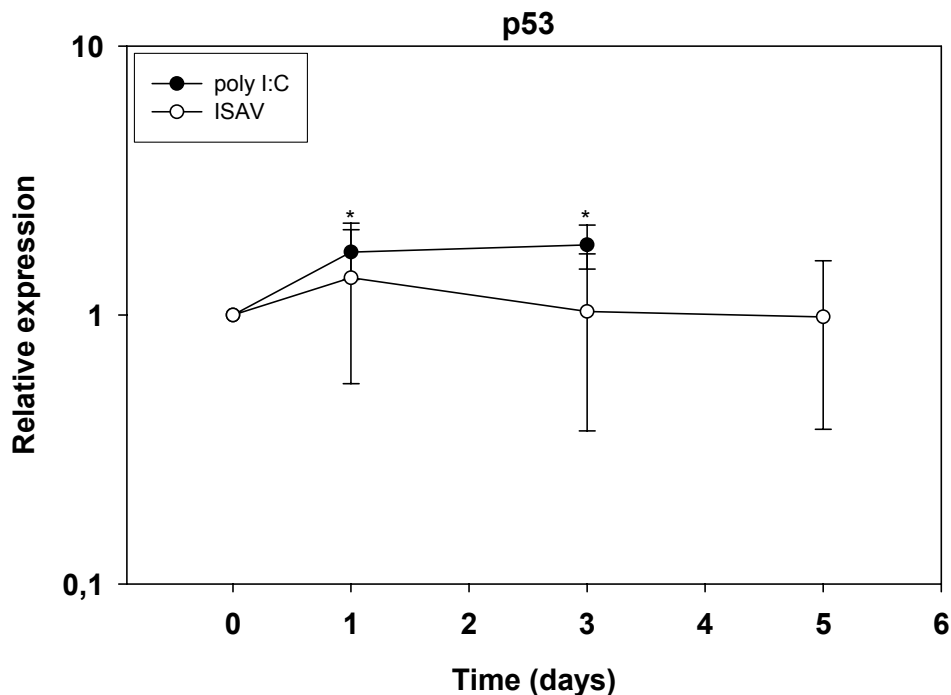
Fig 6.19: Relative expression of p53 in ASK cells. p53 can induce the expression of genes involved in cell cycle arrest and apoptosis. Expression is calculated in REST relative to mock-infected cells and normalized to 18S and EF1-α. (* = statistic significant result)

<u>Relative expression of p62 and p53:</u>

In figure 6.18 we can see that p62 was up-regulated in both poly I:C stimulated and ISAV infected cells. At day 1 after infection/stimulation there was a 3 fold up-regulation in both experiments. In poly I:C stimulated cells the relative expression of p62 was stable from day 1 to day 3. In ISAV infection there is an increase in p62 expression up to a 10 fold up-regulation. The same expression level was observed in day 5 as in day 3. This result indicates that p62 was up-regulated in both poly I:C stimulated and ISAV infected cells, but to a lesser extend in poly I:C stimulated cells.

p53 was up-regulated 2 fold in poly I:C stimulated cells both at day 1 and 3 after stimulation, as shown in figure 6.19. In ISAV infected cells where there was no net up-regulation of p53. This indicates that poly I:C stimulation can induce a up-regulation of p53 compared to ISAV infected cells, where there was hardly any up-regulation.
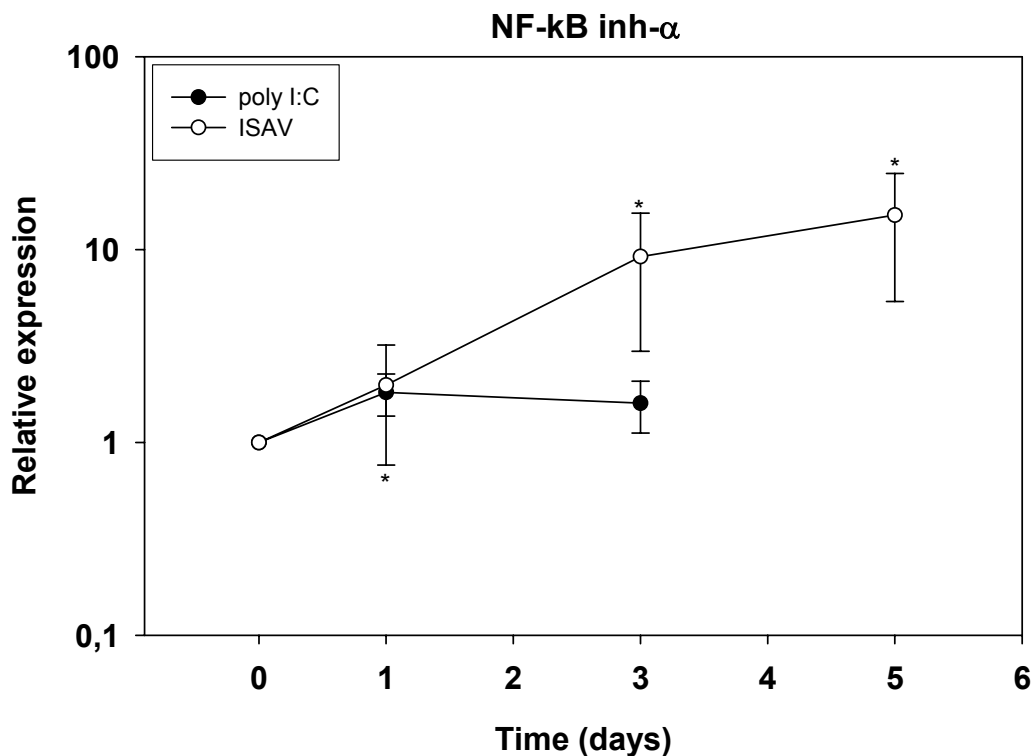
Fig 6.20: Relative expression of NF-kB-inh. in ASK cells. NF-kB is a transcriptionfactor that is an mediator in the immune- and inflammation response.Expression is calculated in REST relative to mock-infected cells and normalized to 18S and EF1-a.(* = statistic significant result)

Relative expression of NF-κB inhibitor-α3

NF-κB is a transcription factor, and is important in immune- and inflammamatory responses. As can be seen in figure 6.20, there was a significant up-regulation of NF-κB inhibitor-α in ISAV infected cells. At day 1 there was a 2 fold up-regulation, and this increased steadily to a 10 fold up-regulation at day 5 post infection. There was a no significant up-regulation of NF-κB inhibitor-α in poly I:C stimulated cells .
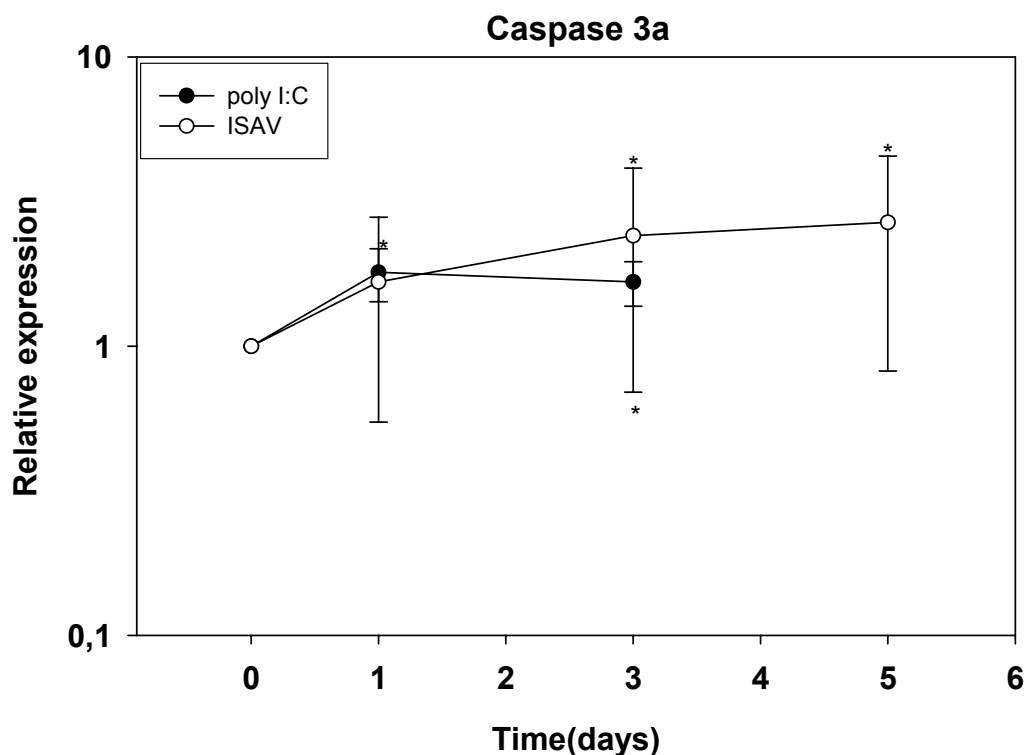
Fig 6.21: Relative expression of caspase-3 in ASK cells. Caspases play an important role in regulating apoptosis. Caspase 3 can activate other caspases. Expression is calculated in REST relative to mock-infected cells and normalized to 18S and EF1-α. (* = statistic significant result, p = 0.05)
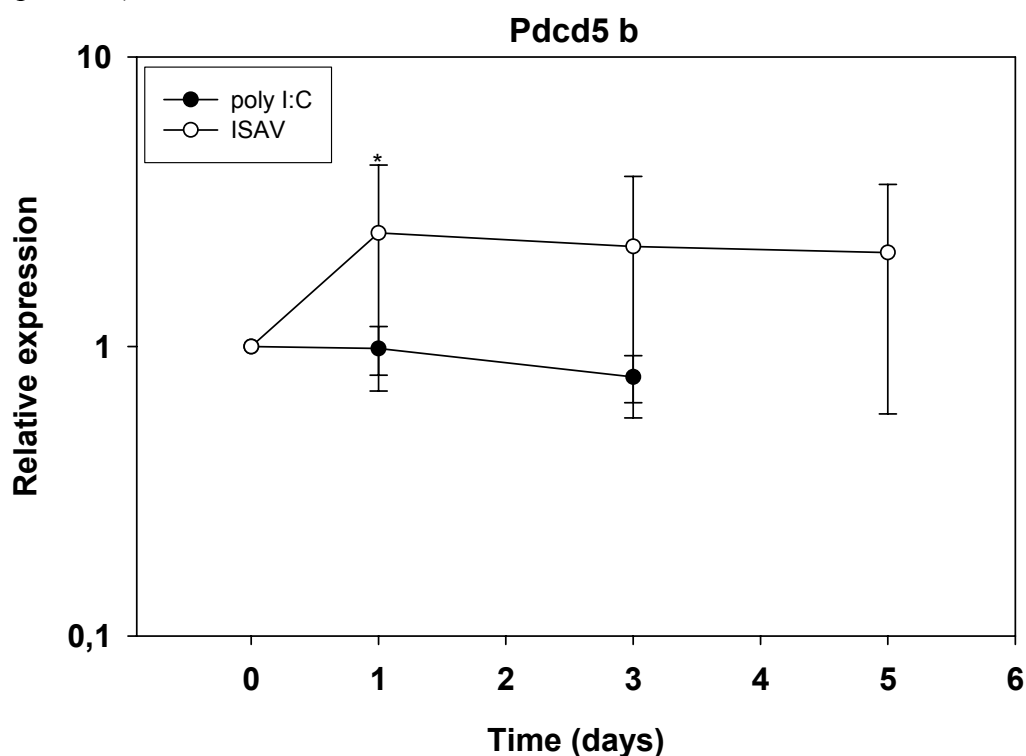
Fig 6.22: Relative expression of Pdcd5b in ASK cells. Programmed cell death 5 (PDCD5) protein is related to regulation of cellular apoptosis. Expression is calculated in REST relative to mock-infected cells and normalized to 18S and EF1-α. (* = statistic significant result

Relative expression of caspase-3 and Pdcd5b:

Both caspase-3 and programmed cell death 5 (Pdcd5) protein is important in cellular apoptosis. As shown in figure 6.21, caspase-3 was up-regulated in both poly I:C stimulated and ISAV infected cells. At day 1 caspase-3 is up-regulated about 3 fold in both experiments. In ISAV infected cells the expression of caspase-3 increased steadily to a 5 fold up-regulation at day 5 post infection. In poly I:C stimulated cells the expression did not change significantly from day 1 to day 3 post stimulation. This results shows that there was an up-regulation of caspase-3 in both poly I:C stimulated and ISAV infected cells.

Pdcd5b was up regulated by ISAV infection at day 1 p.i., but was unchanged in the other samples.

Fig 6.23: Relative expression of IAP-1 in ASK cells. Inhibitor of Apoptosis (IAP) proteins are shown to be involved in suppressing the host cell death response to viral infection. Expression is calculated in REST relative to mock-infected cells and normalized to 18S and EF1α (* = statistic significant result)

Fig 6.24: Relative expression of Mcl-1 in ASK cells. Myeloid leukemia differentiation protein (Mcl-1) is an immune-relevant gene that belongs to the Bcl-2 family. Expression is calculated in REST relative to mock-infected cells and normalized to 18S and EF1-α. (* = statistic significant result)
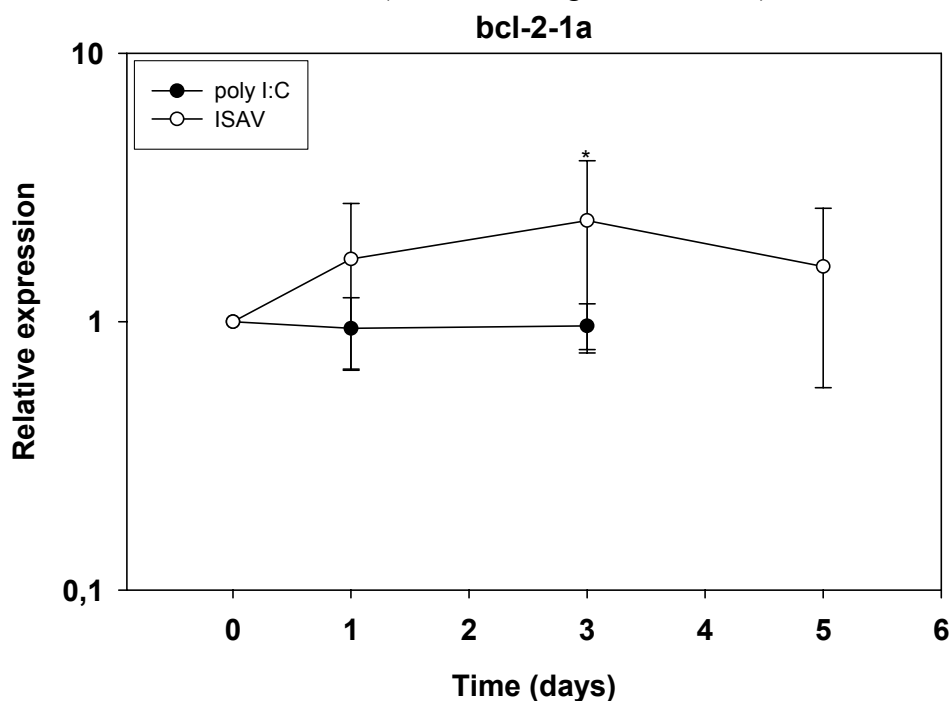
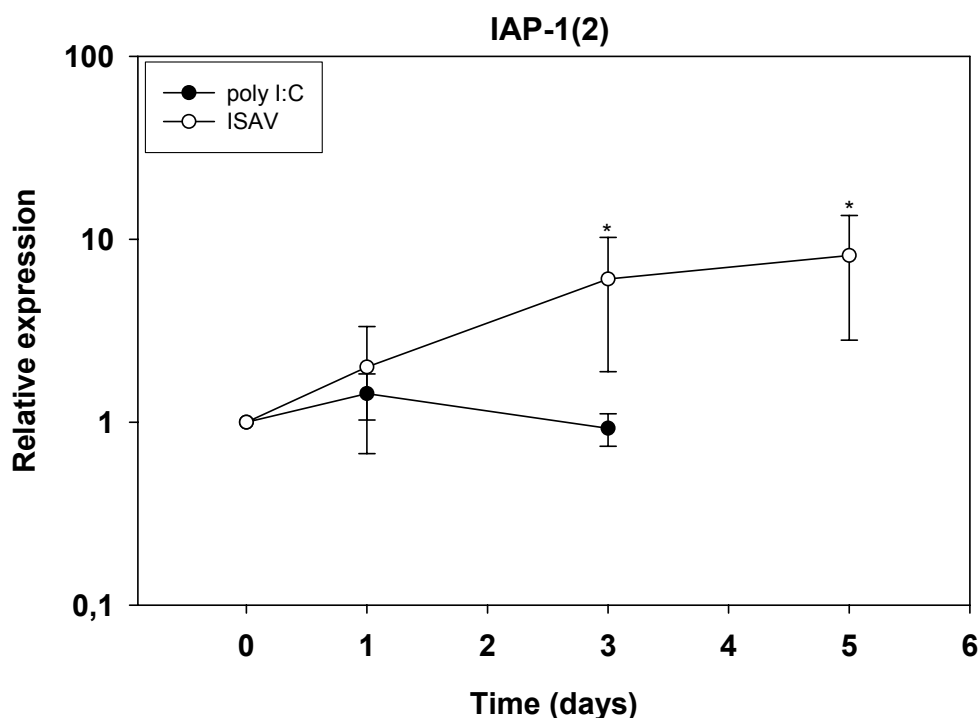**Fig 6.25: Relative expression of Bcl-2 in ASK cells.** The bcl-2 gene encodes a Bcl-X_L-like protein with antiapoptotic effects. Expression is calculated in REST relative to mock-infected cells and normalized to 18S and EF1-α. (* = statistic significant result)
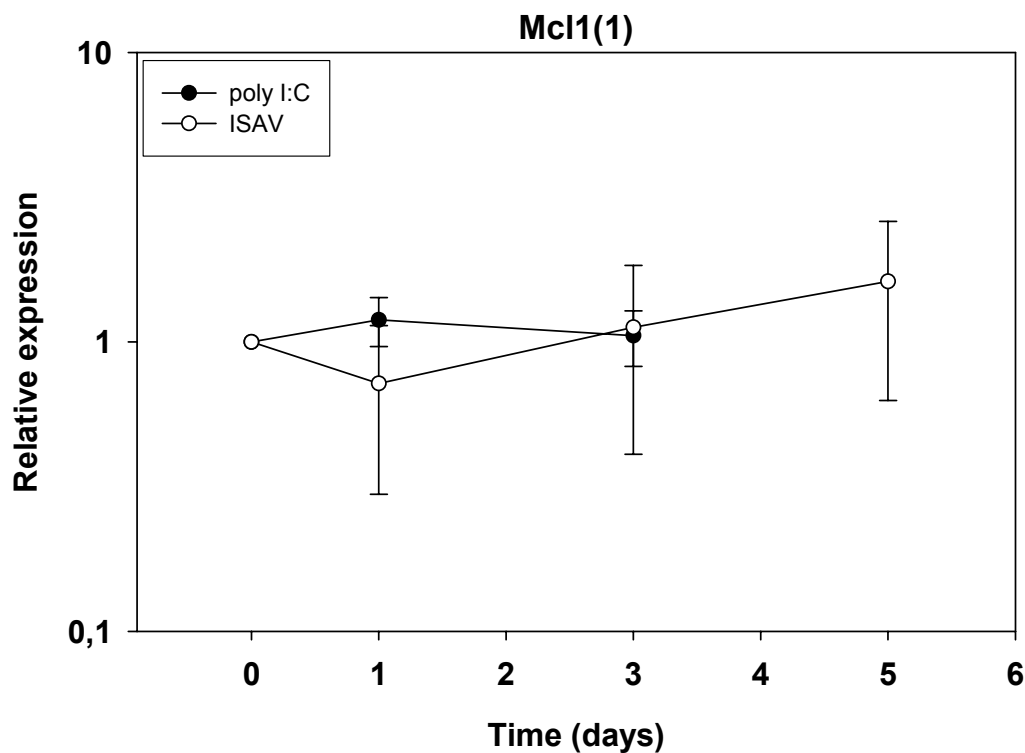
Relative expression of IAP-1, Mcl-1 and bcl-2:

IAP-1, Mcl-1 and Bcl-2 all have antiapoptotic effects in mammalian cells. In figure 6.23 -6.25 we can see that IAP-1 and Bcl2 was up-regulated in ISAV infected cells at day 3 p.i (IAP also at day 5 p.i.). There were no changes after poly I:C treatment of the cells.

Summary of Q-PCR results:

Genes	ISAV	Poly I:C	Genes	ISAV	Poly I:C
IFNα	↑↑↑	↑↑	Galectin-9	↑↑↑	↑↑
Mx	↑↑↑	↑↑	Transal-1	-↓↓	-↓
ISG15	↑↑↑	↑↑	P62	-↑↑	↑↑
OGFr	↑↑↑	↑↑	P53	---	↑↑
IL1-β	-↑↑	--	NF-κB-inh.	-↑↑	↑-
TNFα	-↑↑	--	Caspase-3	-↑↑	↑↑
Hsp90	--↓	-↑	Pdcd5	↑--	--
Hsp70	-↑↑	-↑	IAP-1	-↑↑	--
Cox2	---	↑↑	Mcl-1	---	--
Fip2	--↑	↑↑	Bcl-2	-↑-	--

Table 6.1 : Relative expression of stress related genes. ASK cells infected with ISAV at day 1,3 and 5 p.i. and ASK cells stimulation with poly I:C at day 1 and 3 post stimulation.
↑= significant up-regulation, ↓= significant down regulation, - = not statistic significant

6.3   Gel electrophoresis of amplicons

PCR amplification products should also be tested by agarose gel electrophoresis to ensure primer specificity. All amplicons were run on an agarose gel. Gel electrophoresis results are shown in figure 6.26 and 6.27.
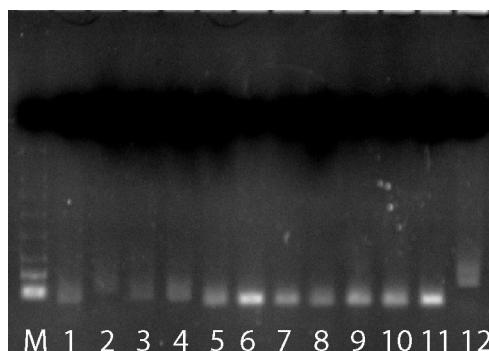
Fig 6.26: Gel electrophoresis of the real-time PCR products of the primers. Lane M contains FlashGel DNA Marker. Lanes 1-12 represent amplification products from 18S, IFNa, Mx, EF1a, hsp90, hsp70, il1b, tnfa, cox2, fip2, ogf1 and isg15, respectively. The agarose gel was visualized under UV-light and picture was taken using Chemi Genius[2] (Bio Imaging System).
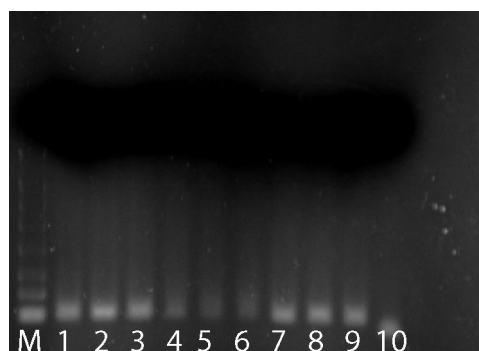
Fig 6.27: Gel electrophoresis of the real-time PCR products of the primers. Lane M contains FlashGel DNA marker. Lanes 1-10 represents amplification products from IAP-1, Pdcd5, Caspase-3, p53, bcl-2, Galectin-9, NF-κB-inh., p62, transaldolase-1 and Mcl-1, respectively. The agarose gel was visualized under UV-light and picture was taken using Chemi Genius[2] (Bio Imaging System).

6.4 Detection of Galectin-9 on a protein level using immunofluorescence and western blotting

A high up-regulation of galectin-9 was observed in both poly I:C stimulated and ISAV-infected cells on a mRNA level. If this protein is produced and secreted from infected cells galectin-9 could be a new marker for ISAV infection in Atlantic salmon (*Salmo salar L*).

6.4.1 Detection of galectin-9 using immunofluorescence-microscopy

ASK cells were seeded in 10 cm^2 six-well tissue-culture plates with 3 coverslips per well and incubated at 20 °C for 2 days as described in earlier chapter. The wells were infected with ISAV (MOI=1) or mock-infected as described earlier. The samples were taken out at day 3 post infection and fixated. The ASK cells were incubated with galectin-9 in three different concentrations (1:50, 1:100 and 1:200). Then the cells were incubated with a secondary antibody, Alexa-Fluor 488 Rabbit anti-goat, which will fluoresce green in immunofluorescence as described earlier. The results are shown in figure:

A: ASK-cells mock infected,
antibody galectin-9 1:50

B: ASK-cells infected with ISAV,
antibody galectin-9 1:50

C: ASK-cells mock-infected,
antibody galectin-9 1:100

D: ASK-cells infected with ISAV,
antibody galectin-9 1:100

E: ASK-cells mock-infected,
antibody galectin-9 1:200

F: ASK-cells infected wth ISAV,
antibody galectin-9 1:200
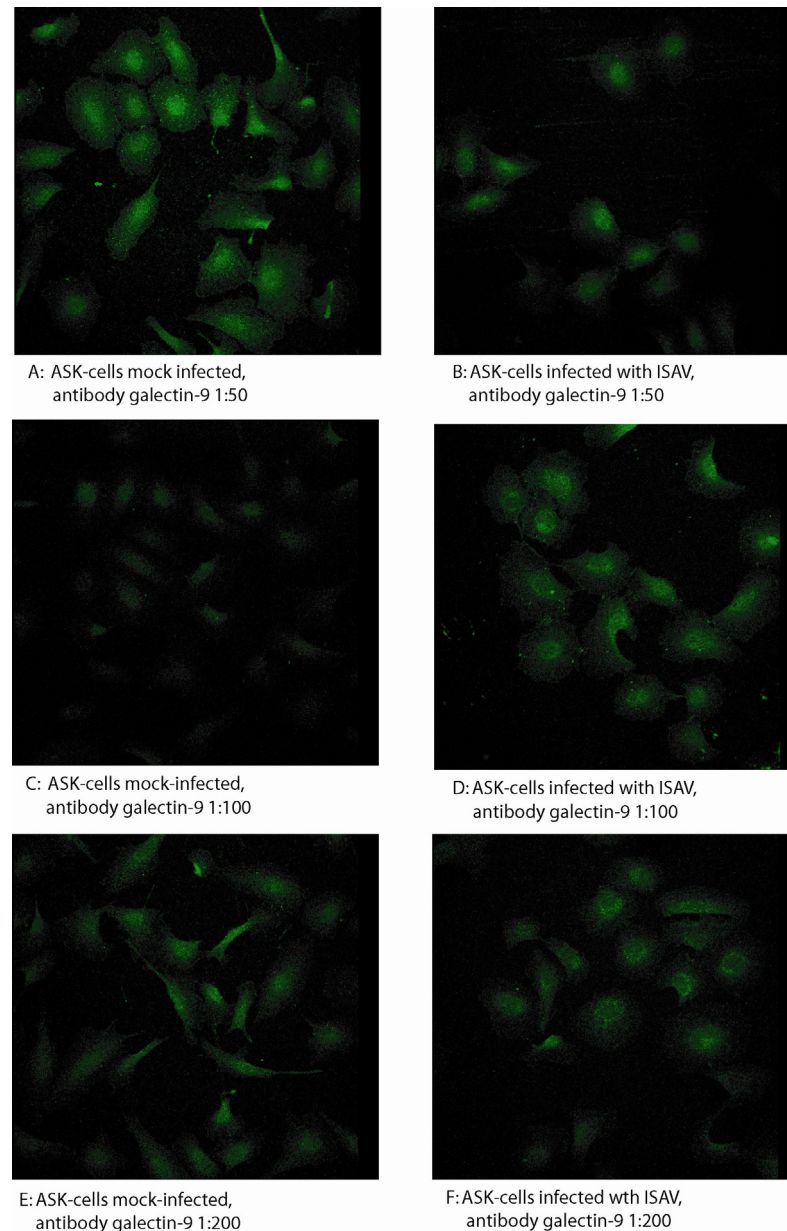
Figure 6.28: Immunofluorescence. Immunofluorescence staining with galectin-9 antibody of ASK cells that either is mock-infected or ISAV infected. The concentration of the antibody used, is shown under each picture.

In figure 6.28, we can see that we have fluorescence in both mock-infected and ISAV infected ASK cells. That means that we have expression of galectin-9 in both infected cells and control cells. In 1:50 dilution (A and B) we have a higher expression of galectin-9 in mock-infected cells compared to ISAV-infected cells.

In 1:100 dilution (C and D) we have a stronger fluorescence in ISAV infected cells compared to mock-infected cells. This can indicate that we have a higher expression of galectin-9 in mock-infected cells.

In 1:200 dilution (E and F) we have almost the same fluorescence in both mock-infected and ISAV-infected cells.

Further, we tried to detect galectin-9 using western blotting, but this was not achieved. Galectin-9 was not detected when the samples were probed with galectin-9 antibody (Not shown).

7   DISCUSSION

The aim of this study was to monitor the expression of different stress-related genes that might be involved in the host defence-mechanisms against ISAV infection. Such analysis of genes induced by ISAV *in vitro,* may help us to better understand the pathogenesis of ISAV in Atlantic salmon. To test this, we used RT-PCR to measure the relative mRNA level of these genes after ISAV infection and poly I:C stimulation. ASK cells infected with ISAV was compared with cells stimulated with poly I:C (polyriboinosinic-polyribocytidylic acid). Poly I:C is widely used as synthetic dsRNA analog *in vivo* and *in vitro* and has also been demonstrated to induce antiviral responses in Atlantic salmon. When we compare these two stress situations with each other we may better understand the innate immune responses during viral infections in this commercially important fish species.

7.1   The *in vitro* infection model

First it was important to monitor the viral replication/infection and to be sure that our viral preparations were able to infect and replicate in ASK cells *in vitro*. This was verified using immunofluorescence. To monitor the infection we used the monoclonal antibody Falk 3H6F8, which is directed to the hemagglutinin-esterase protein on the surface of the ISA-virus (Falk et al., 1998). This antibody has shown to work well in immunofluorescence, and is currently used in diagnostics of ISAV infection. In our hands, this antibody proved highly specific, and there was no detection of immunofluorescence in the mock-infected cells Furthermore, we observed that about 80 % of the cells were infected with ISAV after day 1 p.i., and at day 3 about all of the cells were infected. These results indicate that the ASK cells are infected, and that the virus is replicating. These results therefore confirmed previous observations about the suitability of this antibody in ISAV diagnostics.

In recent years, two more antibodies directed against ISAV have been described, the 6AD/9Z antibody and the whole-virus antibody. Both antibodies are polyclonal but there were no published reports on their suitability in immunofluorescence microscopy. We therefore wanted to evaluate the applicability of these antibodies in immunofluorescence.

As already mentioned, 6AD/9Z is a polyclonal antibody which is directed to a peptide sequence of the hemagglutin-esterase protein in ISAV (Krossoy et al., 2001). This antibody is an anti-serum made in rabbit. Since both the Falk-antibody (3H6F8) and 6AD/9Z is directed

to hemagglutinin-esterase in ISAV the staining pattern would be expected to be roughly similar for these two antibodies. In the immunofluorescence pictures, we observed that there was fluorescence in both mock-infected and ISAV infected cells. This indicates that this antiserum also binds to host cellular proteins, and therefore is not suitable in diagnostics of ISAV infection in Atlantic salmon.

The specificity of the 6AD/9Z antiserum could probably be increased by affinity purification using the peptide from the HE protein (Krossoy et al., 2001). However, if this antiserum mainly recognizes only linear epitopes from HE (positive western signal) it will have limited value in IF, where mainly conformational epitopes are present.

Furthermore, in the specificity testing of the whole-virus antibody we saw the same unspecific binding as seen with the 6AD/9Z antibody. In the immunofluorescence pictures there was fluorescence in both ISAV infected and mock-infected cells. This indicates that the whole-virus antibody is bound unspecific to proteins in the cell. Even though there is a difference in the fluorescence intensity, we could not discriminate between infected and noninfected cells. The Falk- antibody is therefore still superior for diagnostics of ISAV.

The two rabbit antisera were also tested in western blotting. The Falk-antibody (3H6F8) has been tested in western blotting before, but with no luck. The tubulin antibody confirmed that we used about the same amount of protein in all of the samples.

When the samples were probed with 6AD/9Z we observed bands at day 3 and day 5 post infection. This protein migrated at approximately 42 kDa, corresponding to the size of ISAV hemagglutinin esterase. This demonstrated expression of ISAV proteins in the infected cell. This is a very specific binding, because as can be seen from the blot, we do not have any expression of detectable proteins in the control sample. This is in agreement with other published reports using this antiserum (Krossøy et al, 2001).

When the samples were probed with the whole-virus antibody we observed protein-expression at day 1, 3 and 5 post infection. This antibody can recognise different peptides of the ISAV-proteins, and this is why we got expression of proteins of different size. At day 1 p.i. there is only a band around 75 kDa. This is probably the ISAV polymerase (PA), which has a size of 71 kDa. At day 3 p.i. there was also a band at approximately 42 kDa. This indicates expression of the ISAVs HE-protein. Furthermore, expression of a protein around 25 kDa at day 5 p.i., is probably the Matrix protein (M1). This indicates that the whole-virus

antibody mainly recognize polymerase (PA), hemagglutini-esterase (HE) and Matrix-protein (M1) of ISAV.

The explanation for the differences in protein expression at day 1, 3 and day 5 p.i. was probably that protein level was too low only 1 day p.i. It can take some days before the protein-level is high enough to be detected in immunoblotting. In day 1 p.i. it is only polymerase that was sufficiently expressed to be detected. This indicates that the virus has started to replicate, since the amount of polymerase in the added ISA-virus is too low for detection. We are therefore seeing viruses that are replicating, and not only the viruses that we have infected the cells with.

In the control lane we saw a faint signal that was most probably a result leakage from the neighbouring lane. This result indicates that we can use this antibody in western blotting to detect if there is an ISAV infection or not in different samples.

The reason that we got better results when using these antibodies in western blotting, can be that western blotting analyse denatured proteins. This means that the proteins are unfolded and are more available to be detected. The peptide antibody would bind more specific compared to the protein antibody (Falk-antibody), because the Falk-antibody would only detect the protein when it is folded.

These results indicate that the Falk-antibody is the best antibody to be used in diagnostics in immunofluorescence, and the other two antibodies, 6AD/9Z and whole virus antibody can be used in western blot to detect ISAV infection in Atlantic salmon.

7.2 Gene expression in ASK cells after ISAV infection and stimulation with poly I:C- a comparison

ISAV is causing severe disease in Atlantic salmon, but the mechanisms for these effects are not yet fully understood. What is known is that viruses can induce different stress responses in the cell. In a previous study, (Jorgensen et al., 2007) there was observed an up-regulation of different genes that may be involved in antiviral responses, notably genes involved in antigen processing and presentation. Interferon responses, different cytokines and even apoptotic related genes were affected after the infection. By comparing poly I:C stimulated and ISAV infected cells we can see what stress-genes the ISA-virus is inducing. Is the induction of these stress genes beneficial for the host or the pathogen?

The mRNA expression was normalized to two different housekeeping genes, 18S and EF1α. The 18S expression level stayed stable throughout the infection, indicating that the virus infection not affected the 18S expression. The EF1α expression level is down regulated a 2-fold at day 5 p.i. indicating that the infection could affect general gene expression. The 18S-level stayed relatively stable, the ISAV infection has not completely taken over the cells RNA-synthesis.

7.2.1 Interferon-response

Characterization of the teleost IFN system is in its infancy (Robertsen, 2006). Teleosts seem to have one functional type I IFN with highest homology to IFN-α, which has been cloned from Atlantic salmon. Both IFN-α, Mx and ISG15 have been cloned and sequenced in Atlantic salmon, indicating that the interferon-system in Atlantic salmon is similar humans. The interferon system provides a powerful and universal intracellular defence mechanism against viruses. Among the first steps in the innate immune response to viral infections is the induction of interferon responsive genes mediated by the secretion of type I IFN.

As expected, both poly I:C (dsRNA) and ISAV induced a strong IFN-α response. This has been observed previously both *in vitro* and *in vivo* (Jorgensen et al, 2006.) and (Kileng et al., 2007). In the study of Jorgensen, there was the same tendency in up-regulation of IFN-α as presented in this study.

In poly I:C stimulated cells the IFN-α expression was up-regulated at day 1 p.i., but decreased at day 3 p.i. This decrease was observed for several of the genes that were initially

induced. As the concentration of poly I:C in this study was rather high, it may have had a toxic effect on the cells after 3 days of incubation.

Induction of Mx was more rapid compared to IFN-α in ISAV-infected cells. A 10-fold up-regulation of IFN-α led to a 50 fold up-regulation of Mx, which indicates that Mx is very sensitive to low levels of type I IFN. This induction of Mx has been shown in earlier studies (Jorgensen et al., 2007). Among the known interferon-induced antiviral mechanisms, the Mx pathway is one of the most powerful.

Isg15 is one of the earliest and most predominant proteins induced in mammals following IFN-α/β stimulation, which suggest that it has an important function in the interferon system. Our result indicates that ISG15 is highly up-regulated in both ISAV infected cells and after poly I:C stimulation. An up-regulation of ISG15 has been shown earlier in Atlantic salmon infected with other viruses (IPNV) (Rokenes et al., 2007). Analysis revealed strong induction by both poly I:C and by viral infection, and it was suggested that Isg15 has a role in the antiviral interferon response of Atlantic salmon.

The induction of expression of Mx and Isg15 is probably combined effect of the ISAV infection and the interferon secretion. To discriminate between either of these responses, we would need to analysed cells with defects in the interferon system (IFN receptor or STAT negative cells).

These results shows that ISAV is a powerful inducer of key genes of the type I interferon system of Atlantic salmon. Several studies have demonstrated the importance of the innate immune response against virus infection in teleost fish. However, ISAV does not seem to be sensitive to the antiviral action of type I IFN or Mx in Atlantic salmon (Jensen and Robertsen, 2002).

In a study in ISAV infected cells it was demonstrated that that recombinant Atlantic salmon IFN-α did not protect salmon cells against ISAV infection. These data suggests that ISGs including Mx and ISG15 have no antiviral effect against ISAV in Atlantic salmon and that ISAV must have developed mechanisms to counteract the activity of IFN-induced antiviral proteins (Kileng et al., 2007).

Viruses have evolved numerous strategies to specifically counteract and evade host's antiviral responses. Such viral immune evasions mechanisms include production of proteins that inhibit antigen presentation, cytokine production or/and apoptosis.

An IFN-signaling antagonistic effect of the ISAV NS1 protein has been suggested (McBeath et al., 2006). An interferon antagonist protein encoded by segment 7 of ISAV was demonstrated by inhibition of transcription from the Mx promotor. This protein's interfering activity is on the action rather than the production of IFN, since it interferes with the transcription of the antiviral gene Mx.

This result indicates that IFN-α and interferon-induced genes is highly expressed after an ISAV infection, but it seems like that the virus has different mechanisms that counteract the host's immune response. What this mechanism is not yet figured out; it is still to be found out.

7.2.2 Cytokines and stress-response

Different cytokines and stress-genes can be expressed after an ISAV infection. These stress-responses can induce apoptosis of the infected cell. We have investigated different genes that have been found up-regulated during various stress responses in Atlantic salmon.

Interleukin-1β is a pro-inflammatory cytokine released after virus infection. We observed that Il-1β was induced after an ISAV infection, and not in poly I:C stimulated cells. Expression of IL-1β has been analysed in Atlantic salmon during the smolting period (Ingerslev et al., 2006). In an *in vivo* infection trial in Atlantic salmon, ISAV infection increased IL-1β expression, but no effect was seen in fish infected with IPNV. This indicates that IL-1β expression is important both *in vitro* and *in vivo* after an ISAV infection. Therefore it is likely that IL-1β has a role during ISAV infection. Although not extensively studied, there are a few reports of IL-1β involvement in viral infections, including reports of influenza A causing an increase in IL-1β mRNA expression, but also inhibiting the release of IL-1β (Pirhonen et al., 1999) (Ferko et al., 2004). In fish, a study using RT-PCR showed increased IL-1β transcription in rainbow trout infected with viral haemorrhagic septicemia virus (VHSV) (Tafalla et al., 2005). Similarly, rainbow trout infected with infectious

haematopoietic necrosis virus (IHNV) also showed a considerable up-regulation of the IL-1β gene in the spleen (Purcell et al., 2004). The mechanism for cytokine induction after ISAV infection is not known. But in influenza virus infection the most widely postulated trigger for cytokine induction is dsRNA intermediates produced during replication. The mechanism which dsRNA triggers production of cytokines is still not well understood. Recent work implicates Toll-like receptor (TLR) 3 as a sensor for dsRNA in the initiation of an innate immune response. This mechanism can also be the explanation of cytokine release in ISAV infected cells. But further study is required to fully elucidate the involvement of Toll-like receptors and IL-1β in ISAV infection.

TNF-α is a pro inflammatory cytokine that can be induced after virus infection. Expression of TNF-α has been investigated in Atlantic salmon during the smolting period, and was not induced to the same degree as cox-2 and IL-1β (Ingerslev et al., 2006). We found that TNF-α was highly up-regulated during ISAV infection and not after poly I:C stimulation, especially at day 3 and 5 p.i. This indicates that ISAV has mechanisms that induce TNF-α mRNA expression. However, the Ct-values for TNF-α was very high, indicating that TNF-α gene expression is relatively low. In an *in vivo* infection trial of Atlantic salmon there was no induced expression of TNF-α following infection with either IPNV or ISAV (McBeath et al., 2007). This is in contrast with results from another study that showed a strong TNF-α induction after IHNV infection (Purcell et al., 2004). TNF-α is often associated with type I IFN production following a viral infection. However, there are examples of infections with a high induction of a systemic IFN without a significant TNF-α response. These results indicate that TNF-α may be involved after an ISAV infection, but further studies have to be done to look at the involvement of this cytokine in ISAV infection.

NF-κB inhibitor alpha-3 inhibits NF-κB by complexing with and trapping it in the cytoplasm. It may be involved in regulation of transcriptional responses to NF-κB, including cell adhesion, immune and proinflammatory responses, apoptosis, differentiation and growth. NF-κB inhibitor alpha-3 is controlled by sequential serine-phosphorylation, ubiquitination and degradation.

NF-κB is an important transcription factor that is involved in the expression of immune- and inflammatory genes. NF-κB is a transcription factor, whose activation is important in infections by viral pathogens including influenza viruses. Because gene expression of many pro-inflammatory and antiviral cytokines is controlled by this factor, the

concept emerged that NF-κB and its upstream regulator IκB kinase are essential components of the innate antiviral immune response to infectious pathogens. The inhibitor gene was found up-regulated after ISAV infection in a microarray trial. We observed that NF-κB inhibitor alpha 3 was highly up regulated in ISAV infected cells and not in poly I:C stimulated cells. This can indicate that ISAV modulate the NF-κB signalling pathway via dsRNA independent mechanisms. A general view has been that NF-κB is an essential component of the innate antiviral immune response to infectious pathogens, but there has also been reported that NF-κB activity promotes efficient virus production in influenza (Wurzer et al., 2004). NF-κB can act both proapoptotically and provirally in the context of an influenza virus infection. NF-κB itself has not been cloned or sequenced in Atlantic salmon and we have therefore not analysed this factor itself.

Opioid growth factor receptor was up-regulated in both ISAV infected and poly I:C stimulated cells, but to a higher extend in ISAV infected cells. Ogfr has been cloned and sequenced in Atlantic salmon. In a study were Atlantic salmon was infected with *Gyrodactylus salaris,* a high up-regulation of Ogfr in two out of six susceptible fish was observed, which may indicate that its expression was localised to sites of wounds resulting from a heavy burden of *G.salaris (Matejusova et al., 2006).* Ogfr has not been studied in ISAV infected cells before, but the result obtained from this study indicates that Ogfr can be involved in the immune response against ISAV in Atlantic salmon. Another possible explanation can be that is induced by ISAV, so that the cells are protected.

Heat shock protein 90 (Hsp90) expression was not affected in poly I:C or ISAV infected cells.

Heat shock proteins are remarkably conserved in all living organism, and their expression is induced in response to a variety of physiological and environmental insults, like virus infections. In the cytosol these proteins play an essential role as molecular chaperones by assisting the correct folding of misfolded proteins, preventing protein aggregation, transport of proteins, and supporting antigen processing and presentation. In contrast, membrane-bound or extracellularly located HSPs act as danger signals and elicit immune responses mediated either by the adaptive or innate immune system. In human cancer cells hsp70 has been detected in the cell membrane, indicating that it has an immunological effect (Multhoff, 2007).

In our study hsp70 was highly up-regulated in ISAV infected cells compared to poly I:C stimulated cells. Since it was not up-regulated in poly I:C stimulated cells, it may indicate that ISAV affect the expression of hsp70 in a dsRNA independent way. Neither hsp70 nor other HSPs has been cloned and expressed in Atlantic salmon before. This gene was found interesting, because there was a change in gene expression of hsp70 after a micro-array trial (sequence based on rainbow trout hsp70).

As can be seen in the cox-2 expression graph, cox-2 expression was highly up-regulated in poly I:C stimulated cells compared to control samples, but not in ISAV infected cells. This can indicate that ISAV is suppressing the cox-2 expression. Cox-2 has been cloned in Atlantic salmon (Ingerslev et al., 2006), and is constitutively expressed in kidney cells during the smoltification period of Atlantic salmon. The influence of cox-2 within the immune system is via synthesis of prostaglandins that are important in all stages of inflammation. This result can indicate that ISAV has mechanisms that suppress Cox-2 expression in ASK-cells.

A Fourteen Kilodalton Interaction Protein 2(FIP2)-like gene has been isolated from Atlantic salmon (Collins et al., 2007), and this gene is up-regulated following infection with the parasite *Gyrodactylus salaris.* This gene is thought to have a role in TNFα cytolysis and in cellular remodelling. FIP2 can also work as an inhibitor of apoptosis. We found that FIP2 initially was more induced in poly I:C stimulated than ISAV infected cells. This can indicate that ISAV suppresses the expression of FIP2. There has been found elevated levels of FIP2 following mechanical wounding in rat pulpal cells (Oyama et al., 2005). The relatively high dose of poly I:C used in this study may therefore explain our findings.

P62 is a gene that can be induced after stress, like a virus infection. Paramyxoviruses, use ubiquitin in their budding process and sequestosome 1/p62 is involved in the ubiquitin proteasomal degradation pathway. When cells are infected with canine distemper virus, p62 expression is induced (Selby et al., 2006).

P62 has not been cloned and sequenced in Atlantic salmon, but it was shown to be up-regulated in a microarray trial. P62 is up-regulated after ISAV infection, and not poly I:C stimulation. This can indicate that p62 is induced by dsRNA independent mechanisms. The role of p62 in ISAV infection is not clear but involvement in regulation of protein aggregation and clearance is an interesting hypothesis.

7.2.3 Apoptosis

Apoptosis is a genetically controlled process of cell suicide in response to a variety of stimuli. Apoptosis is considered a part of the innate immune response to virus infection, limiting the time and cellular machinery available for viral replication (Thomson, 2001). There are several ways by which viruses might activate the apoptotic pathway. Some viruses do so through direct action of specific viral proteins, such as adenovirus E1A protein. Other viruses induce apoptosis indirectly through their effects on cellular functions, for example by shutting down protein synthesis (Hasnain et al., 2003) or by activating pro-apoptotic proteins, such as up-regulation of Fas which can be activated by the dsRNA-activated PKR during influenza virus infection (Balachandran et al., 2000).

A recent study has indicated that the mechanism of cell death during ISAV infection is cell type-specific. In SHK-1 cell line, ISAV induces apoptosis, but in TO cell lines the virus infection leads to necrosis (Joseph et al., 2004).

It has been indicated that during infection with most RNA viruses, the cells undergo pronounced morphological changes leading to cell rounding. In ASK cell line, which are macrophage-like cell lines, virus replication can lead to cytopathic effect.

As can be seen from the pictures of the aktin-filament staining, ISAV is causing CPE to the ASK cells. In day 5 p.i. we can see that the cells have shrunken, and have a rounder shape.

Two general pathways that are known to cause CPE and eukaryotic cell death during virus infection are apoptosis and necrosis. Cell death in ASK cells is probably due to apoptosis, as can be seen in SHK-1 cell line. There are different genes that may be involved in the regulation of apoptosis.

In general, tumour suppressor protein p53 is an essential component of an emergency stress response that prevents the growth and survival of damaged or abnormal cells. Various stresses, like viral infection, increase p53 transcriptional activity, which induces the expression of genes involved in cell cycle arrest and apoptosis. The induction of apoptotic cell death is a hallmark of influenza virus infection. P53 expression was not up-regulated in ISAV infected ASK cells. In poly I:C stimulated cells there is a slight significant up-regulation of this gene. This can indicate that ISAV suppresses the p53 expression, but it can also indicate that p53 was not induced at all in these cells. ISAV may have mechanisms that suppress apoptosis, so that the cells stay alive much longer, so the virus has enough time to replicate.

P53 has been extensively studied in mammalian models, but relatively little is known about its specific function in lower vertebrates, like Atlantic salmon. Additional studies are necessary to further characterize the regulation of p53 and apoptotic signalling in Atlantic salmon.

Galectin-9 is highly expressed in both poly I:C stimulated and ISAV infected ASK cells. Galectin-9 has been found up-regulated after poly I:C stimulation in human vascular endothelial cells (Ishikawa et al., 2004). Galectins has a variety of biological functions, such as cell aggregation and adhesion, proliferation, cell death, and modulation of inflammation. Gal-9 induces various biological reactions such as chemotaxis of eosinophils and apoptosis of T-cells (Imaizumi et al., 2007). The most interesting biological function of galectin-9 is its involvement in T-cell apoptosis. Since Galectin-9 is highly expressed on an mRNA level, it may indicate that ISAV can induce T-cell apoptosis. Galectin-9 has not been cloned and sequenced in Atlantic salmon, but this gene was found up-regulated in a microarray trial. It would be interesting to go further with this gene to see if it is also up regulated on a protein level.

As mentioned before, transaldolase is a key enzyme of the reversible nonoxidative branch of the pentose phosphate pathway. It may be involved in protection of the cell from reactive oxygen intermediates, which causes apoptotic cell death (Banki et al., 1996). Therefore, it was interesting to see if transaldolase was involved after an ISAV infection in Atlantic salmon. It is not a gene that has been cloned or characterized in Atlantic salmon, but it was found to be up regulated after ISAV infection in a microarray trial. As can be seen from the transaldolase expression in our study the expression decreases steadily from day 1 post stimulation/infection in both poly I:C stimulated and ISAV infected cells, but to a higher extend in ISAV infected cells. This indicates that transaldolase is down regulated in ISAV infected cells compared to the mock infected cells. This can indicate that ISAV is causing apoptosis in ASK cells, but this has to be further analysed.

There is a slight up-regulation of caspase-3 expression after both poly I:C stimulation and ISAV infection. Caspase is a central player in apoptosis regulation and its activity is often measured to determine the impact of a given apoptotic stimulus. A recent study demonstrated that caspase-3 activation is essential for influenza A virus propagation (Wurzer et al., 2003). On the other hand, Joseph *et al* (2004) has studied ISAV-induced apoptosis and suggested that ISAV-induced apoptosis in Atlantic salmon occurs via the caspase-activation pathway, but

may not involve activation of caspase-3. Caspase-3 has been cloned and characterized in Atlantic salmon (*Salmo salar*) (Takle et al., 2006). The salmon genome contained two genetically distinct variants of salmon caspase-3, caspase-3a and caspase-3b. Even though caspase-3 is up regulated, it is not sure if caspase-3 is involved in apoptosis.

The programmed cell death 5 (PDCD5) protein is a novel protein related to regulation of cell apoptosis (Chen et al., 2001b). In this study they demonstrated that the level of PDCD5 protein expressed in tumor-cells undergoing apoptosis is significantly increased compared with normal cells and the protein translocates rapidly from the cytoplasm to the nucleus of cells during apoptosis. PDCD5 can be an early marker for apoptosis. In ISAV infected cells the PDCD5 expression was not significantly changed.

Inhibitor of apoptosis (IAP) family proteins is suppressors of apoptosis. IAP was first discovered in baculoviruses, and where shown to be involved in suppressing the host cell death response to viral infection (Deveraux and Reed, 1999). IAP is up regulated a 10-fold in ISAV infected cells compared to mock infected cells. On the other hand, in poly I:C stimulated cells there is no up-regulation of this gene. This strongly indicates that ISAV has mechanisms that induce this inhibitor of apoptosis gene. Apoptosis is mainly thought to be a process initiated by the host cell to limit the time and cellular machinery available for viral replication (Thompson, 1995). Many RNA viruses, including ISAV (Joseph et al., 2004), are known to induce apoptosis under conditions which allow efficient virus multiplication. IAP has not been characterized in Atlantic salmon before, but in this study it can indicate that the up-regulation of IAP after ISAV infection might be a mechanism there the virus delay the apoptotic process in the host cell, to ensure more time for viral replication.

Myeloid leukemia differentiation protein (Mcl1) is an immune-relevant gene that belongs to the Bcl-2 family. It is a gene that is important in apoptosis. The salmon Mcl-1 amino acid sequence shared significantly high similarities with human, mouse, zebra fish and several other isoforms of this gene, particularly in the portion close to the C-terminus. A similar function as antiapoptotic regulator might be considered for the salmon Mcl-1 isoform. There has been shown an up-regulation of Mcl-1 in Atlantic salmon (*Salmo salar L*) infected with *Gyrodactylus salaris* in the skin-cells of the salmon (Matejusova et al., 2006). But Mcl1 was not induced in poly I:C stimulated and ISAV infected cells. This indicates that Mcl1 is not involved in apoptosis of ASK cells after ISAV infection.

Finally, there was observed a slight up-regulation of bcl-2 after ISAV infection. This was only seen at day 3 p.i. The bcl-2 gene encodes a Bcl-X_L -like protein with antiapoptotic effect. Bcl-2 can affect glycosylation pathways, and can function in two distinct ways to inhibit influenza virus induced cell death. It can also alter influenza virus yield and spread. (Olsen et al., 1996). But since there was only a slight up regulation of bcl-2 after the ISAV infection, it can indicate that bcl-2 is not an important apoptotic gene during ISAV infection.

From the mRNA expression trial, it is clearly that ISAV induces various responses in the cells. But even though we have an expression of these genes on an mRNA level, it is not certain that it is expressed on a protein level. It is the proteins that exert the biological functions in the cells. This is just an indication of possible genes that are involved after ISAV infection. To see if these up regulation can induce a biological function, there has to be done functional studies of these genes in Atlantic salmon.

7.3   Galectin-9 expression on a protein level

Even though we can see a high up-regulation of galectin-9 after an ISAV infection on a mRNA level, it might not have a biological impact on the cell, because it is the proteins that exert the effects in the cells. The up regulation of galectin-9 is an interesting finding, because of its involvement in T-cell apoptosis, so an up-regulation on a protein level could indicate that ISA have mechanisms that are involved in T-cell apoptosis.

We therefore wanted to observe the galectin-9 expression on a protein level after an ISAV infection. To do this, we used immunofluorescence as shown in figure 7.26, in the result section. Cells both infected with ISAV and mock-infected were probed with a galectin-9 antibody. Galectin-9 antibody is an affinity purified goat polyclonal antibody raised against a peptide mapping near the C-terminus of galectin-9 of human origin.

We expected to find more galectin-9 expressed in the ISAV infected cells compared to the mock-infected cells. But as can be seen from the pictures, this was not the case. When the antibody was used in a dilution of 1:50 (A and B), it appeared that there was a higher expression of galectin-9 in the mock-infected cells. In an antibody dilution 1:100 (C and D) it

can look like it is in the opposite way, that there is a higher expression of galectin-9 in the infected cells compared to the control. This probably means that this galectin-9 antibody is unsuitable in immunoflourescence on salmon cells. One of the reasons that this antibody don't work well in Atlantic salmon can be that we have used an antibody that made against the human galectin. We are not sure if this antibody recognizes galectin in salmon, because the galectin-protein could differ in sequence. This in one of the problems when studying Atlantic salmon cells in immunofluorescence. There are few antibodies available, and the ones that are available are often made against human proteins.

Furthermore, it is not sure that even though we have a high mRNA expression level of galectin-9, it is produced more galectin-9 on a protein level. Galectin-9 can also already have been secreted from the cell, so galectin-9 can not be detected by immunofluorescence.

The induction of gal-9 can be important for ISAVs pathogenesis in Atlantic salmon, because T-cells are very important in the immune response of the host cell. But this has to be studied further, and to do so it probably has to be a made a more specific antibody directed to the galectin-9 protein in Atlantic salmon. This observation can be a step further in understanding the pathogenesis of ISAV in Atlantic salmon.

8 CONCLUSION

In conclusion, we have found that many different stress-related genes are up-regulated after ISAV infection in Atlantic salmon, as shown in table 6.1, in section 6.2. This induction can either be dsRNA-induced or dsRNA-independent, indicating that ISAV may have different mechanism to induce stress-related genes, which may be involved in the host immune-response. This induction can either be beneficial for the host or the pathogen. The ISA-virus is causing many responses in the cell, and this may eventually lead to cell death, apoptosis.

Even though we have seen an up-regulation on an mRNA level, it might not have a biological effect on the cell. It is the proteins that exert the biological functions in the cell. But these results can give an indication on what effects the ISA-virus has on the cells in Atlantic salmon. This study has led us to a better understanding of the complex pathogenesis of ISAV.

9 REFERENCES

ADAMS, J. M. & CORY, S. (1998) The Bcl-2 protein family: arbiters of cell survival. *Science,* 281**,** 1322-6.

ALEXOPOULOU, L., HOLT, A. C., MEDZHITOV, R. & FLAVELL, R. A. (2001) Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature,* 413**,** 732-8.

ARYA, M., SHERGILL, I. S., WILLIAMSON, M., GOMMERSALL, L., ARYA, N. & PATEL, H. R. (2005) Basic principles of real-time quantitative PCR. *Expert Rev Mol Diagn,* 5**,** 209-19.

ASPEHAUG, V., MIKALSEN, A. B., SNOW, M., BIERING, E. & VILLOING, S. (2005) Characterization of the infectious salmon anemia virus fusion protein. *J Virol,* 79**,** 12544-53.

BALACHANDRAN, S., KIM, C. N., YEH, W. C., MAK, T. W., BHALLA, K. & BARBER, G. N. (1998) Activation of the dsRNA-dependent protein kinase, PKR, induces apoptosis through FADD-mediated death signaling. *Embo J,* 17**,** 6888-902.

BALACHANDRAN, S., ROBERTS, P. C., BROWN, L. E., TRUONG, H., PATTNAIK, A. K., ARCHER, D. R. & BARBER, G. N. (2000) Essential role for the dsRNA-dependent protein kinase PKR in innate immunity to viral infection. *Immunity,* 13**,** 129-41.

BANKI, K., HUTTER, E., COLOMBO, E., GONCHOROFF, N. J. & PERL, A. (1996) Glutathione levels and sensitivity to apoptosis are regulated by changes in transaldolase expression. *J Biol Chem,* 271**,** 32994-3001.

BIERIN, E., FALK, K., HOEL, E., THEVARAJAN, J., JOERINK, M., NYLUND, A., ENDRESEN, C. & KROSSOYL, B. (2002) Segment 8 encodes a structural protein of infectious salmon anaemia virus (ISAV); the co-linear transcript from Segment 7 probably encodes a non-structural or minor structural protein. *Dis Aquat Organ,* 49**,** 117-22.

BJERRUM, O., HEEGARD,N. (2001) Western Blotting. IN JOHN WILEY & SONS, L. (Ed.) *ENCYCLOPEDIA OF LIFE SCIENCES.*

BRYDON, E. W., MORRIS, S. J. & SWEET, C. (2005) Role of apoptosis and cytokines in influenza virus morbidity. *FEMS Microbiol Rev,* 29**,** 837-50.

BUSTIN, S. A. (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol,* 25**,** 169-93.

CAREY, M. A., BRADBURY, J. A., SEUBERT, J. M., LANGENBACH, R., ZELDIN, D. C. & GERMOLEC, D. R. (2005) Contrasting effects of cyclooxygenase-1 (COX-1) and COX-2 deficiency on the host response to influenza A viral infection. *J Immunol,* 175**,** 6878-84.

CHEN, W., CALVO, P. A., MALIDE, D., GIBBS, J., SCHUBERT, U., BACIK, I., BASTA, S., O'NEILL, R., SCHICKLI, J., PALESE, P., HENKLEIN, P., BENNINK, J. R. & YEWDELL, J. W. (2001a) A novel influenza A virus mitochondrial protein that induces cell death. *Nat Med,* 7**,** 1306-12.

CHEN, Y., SUN, R., HAN, W., ZHANG, Y., SONG, Q., DI, C. & MA, D. (2001b) Nuclear translocation of PDCD5 (TFAR19): an early signal for apoptosis? *FEBS Letters,* 509**,** 191-6.

CHIPUK, J. E., KUWANA, T., BOUCHIER-HAYES, L., DROIN, N. M., NEWMEYER, D. D., SCHULER, M. & GREEN, D. R. (2004) Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. *Science,* 303**,** 1010-4.

CLOUTHIER, S. C., RECTOR, T., BROWN, N. E. & ANDERSON, E. D. (2002) Genomic organization of infectious salmon anaemia virus. *J Gen Virol,* 83**,** 421-8.

COLLINS, C. M., OLSTAD, K., STERUD, E., JONES, C. S., NOBLE, L. R., MO, T. A. & CUNNINGHAM, C. O. (2007) Isolation of a FIP2-like gene from Atlantic salmon (Salmo salar L.), found upregulated following infection with the monogenean parasite Gyrodactylus salaris Malmberg, 1957. *Fish Shellfish Immunol,* 22**,** 282-8.

COX, R. J., BROKSTAD, K. A. & OGRA, P. (2004) Influenza virus: immunity and vaccination strategies. Comparison of the immune response to inactivated and live, attenuated influenza vaccines. *Scand J Immunol,* 59**,** 1-15.

DANNEVIG, B. H., FALK, K. & NAMORK, E. (1995) Isolation of the causal virus of infectious salmon anaemia (ISA) in a long-term cell line from Atlantic salmon head kidney. *J Gen Virol,* 76 (Pt 6)**,** 1353-9.

DEGTEREV, A., BOYCE, M. & YUAN, J. (2003) A decade of caspases. *Oncogene,* 22**,** 8543-67.

DEVERAUX, Q. L. & REED, J. C. (1999) IAP family proteins--suppressors of apoptosis. *Genes Dev,* 13**,** 239-52.

DEVOLD, M., KROSSOY, B., ASPEHAUG, V. & NYLUND, A. (2000) Use of RT-PCR for diagnosis of infectious salmon anaemia virus (ISAV) in carrier sea trout Salmo trutta after experimental infection. *Dis Aquat Organ,* 40**,** 9-18.

FALK, K., ASPEHAUG, V., VLASAK, R. & ENDRESEN, C. (2004) Identification and characterization of viral structural proteins of infectious salmon anemia virus. *Journal ofVirology,* 78**,** 3063-71.

FALK, K., NAMORK, E. & DANNEVIG, B. H. (1998) Characterization and applications of a monoclonal antibody against infectious salmon anaemia virus. *Dis Aquat Organ,* 34**,** 77-85.

FALK, K., NAMORK, E., RIMSTAD, E., MJAALAND, S. & DANNEVIG, B. H. (1997) Characterization of infectious salmon anemia virus, an orthomyxo-like virus isolated from Atlantic salmon (Salmo salar L.). *Journal of Virology,* 71**,** 9016-23.

FERKO, B., STASAKOVA, J., ROMANOVA, J., KITTEL, C., SEREINIG, S., KATINGER, H. & EGOROV, A. (2004) Immunogenicity and protection efficacy of replication-deficient influenza A viruses with altered NS1 genes. *J Virol,* 78**,** 13037-45.

FLORY, E., KUNZ, M., SCHELLER, C., JASSOY, C., STAUBER, R., RAPP, U. R. & LUDWIG, S. (2000) Influenza virus-induced NF-kappaB-dependent gene expression is mediated by overexpression of viral proteins and involves oxidative radicals and activation of IkappaB kinase. *J Biol Chem,* 275**,** 8307-14.

FRITSCHY, J.-M. (2001) Immunofluorescence. IN JOHN WILEY & SONS, L. (Ed.) *ENCYCLOPEDIA OF LIFE SCIENCES.*

GEETHA, T. & WOOTEN, M. W. (2002) Structure and functional properties of the ubiquitin binding protein p62. *FEBS Lett,* 512**,** 19-24.

GIBSON, U. E., HEID, C. A. & WILLIAMS, P. M. (1996) A novel method for real time quantitative RT-PCR. *Genome Res,* 6**,** 995-1001.

GROSS, A., MCDONNELL, J. M. & KORSMEYER, S. J. (1999) BCL-2 family members and the mitochondria in apoptosis. *Genes Dev,* 13**,** 1899-911.

HAGMAIER, K., JENNINGS, S., BUSE, J., WEBER, F. & KOCHS, G. (2003) Novel gene product of Thogoto virus segment 6 codes for an interferon antagonist. *J Virol,* 77**,** 2747-52.

HALLER, O., FRESE, M. & KOCHS, G. (1998) Mx proteins: mediators of innate resistance to RNA viruses. *Rev Sci Tech,* 17**,** 220-30.

HALLER, O., STAEHELI, P. & KOCHS, G. (2007) Interferon-induced Mx proteins in antiviral host defense. *Biochimie,* 89**,** 812-8.

HASNAIN, S. E., BEGUM, R., RAMAIAH, K. V., SAHDEV, S., SHAJIL, E. M., TANEJA, T. K., MOHAN, M., ATHAR, M., SAH, N. K. & KRISHNAVENI, M. (2003) Host-pathogen interactions during apoptosis. *J Biosci,* 28**,** 349-58.

HIGUCHI, R., FOCKLER, C., DOLLINGER, G. & WATSON, R. (1993) Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Biotechnology (N Y),* 11**,** 1026-30.

HINSHAW, V. S., OLSEN, C. W., DYBDAHL-SISSOKO, N. & EVANS, D. (1994) Apoptosis: a mechanism of cell killing by influenza A and B viruses. *J Virol,* 68**,** 3667-73.

HIRASHIMA, M., KASHIO, Y., NISHI, N., YAMAUCHI, A., IMAIZUMI, T. A., KAGESHITA, T., SAITA, N. & NAKAMURA, T. (2004) Galectin-9 in physiological and pathological conditions. *Glycoconj J,* 19**,** 593-600.

HSU, D. K., YANG, R. Y. & LIU, F. T. (2006) Galectins in apoptosis. *Methods Enzymol,* 417**,** 256-73.

IMAIZUMI, T., YOSHIDA, H., NISHI, N., SASHINAMI, H., NAKAMURA, T., HIRASHIMA, M., OHYAMA, C., ITOH, K. & SATOH, K. (2007) Double-stranded RNA induces galectin-9 in vascular endothelial cells: involvement of TLR3, PI3K, and IRF3 pathway. *Glycobiology,* 17**,** 12C-5C.

INGERSLEV, H. C., CUNNINGHAM, C. & WERGELAND, H. I. (2006) Cloning and expression of TNF-alpha, IL-1beta and COX-2 in an anadromous and landlocked strain of Atlantic salmon (Salmo salar L.) during the smolting period. *Fish Shellfish Immunol,* 20**,** 450-61.

ISHIKAWA, A., IMAIZUMI, T., YOSHIDA, H., NISHI, N., NAKAMURA, T., HIRASHIMA, M. & SATOH, K. (2004) Double-stranded RNA enhances the expression of galectin-9 in vascular endothelial cells. *Immunol Cell Biol,* 82**,** 410-4.

JENSEN, I. & ROBERTSEN, B. (2002) Effect of double-stranded RNA and interferon on the antiviral activity of Atlantic salmon cells against infectious salmon anemia virus and infectious pancreatic necrosis virus. *Fish Shellfish Immunol,* 13**,** 221-41.

JORGENSEN, S. M., HETLAND, D. L., PRESS, C. M., GRIMHOLT, U. & GJOEN, T. (2007) Effect of early infectious salmon anaemia virus (ISAV) infection on expression of MHC pathway genes and type I and II interferon in Atlantic salmon (Salmo salar L.) tissues. *Fish Shellfish Immunol,* 23**,** 576-88.

JORGENSEN, S. M., KLEVELAND, E. J., GRIMHOLT, U. & GJOEN, T. (2006) Validation of reference genes for real-time polymerase chain reaction studies in atlantic salmon. *Marine Biotechnology (NY),* 8**,** 398-408.

JOSEPH, T., CEPICA, A., BROWN, L., IKEDE, B. O. & KIBENGE, F. S. (2004) Mechanism of cell death during infectious salmon anemia virus infection is cell type-specific. *J Gen Virol,* 85**,** 3027-36.

KATO, H., TAKEUCHI, O., SATO, S., YONEYAMA, M., YAMAMOTO, M., MATSUI, K., UEMATSU, S., JUNG, A., KAWAI, T., ISHII, K. J., YAMAGUCHI, O., OTSU, K., TSUJIMURA, T., KOH, C. S., REIS E SOUSA, C., MATSUURA, Y., FUJITA, T. & AKIRA, S. (2006) Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature,* 441**,** 101-5.

KIBENGE, F. S., MUNIR, K., KIBENGE, M. J., JOSEPH, T. & MONEKE, E. (2004) Infectious salmon anemia virus: causative agent, pathogenesis and immunity. *Anim Health Res Rev,* 5**,** 65-78.

KIBENGE, F. S., XU, H., KIBENGE, M. J., QIAN, B. & JOSEPH, T. (2007) Characterization of gene expression on genomic segment 7 of infectious salmon anaemia virus. *Virol J,* 4**,** 34.

KILENG, O., BRUNDTLAND, M. I. & ROBERTSEN, B. (2007) Infectious salmon anemia virus is a powerful inducer of key genes of the type I interferon system of Atlantic salmon, but is not inhibited by interferon. *Fish Shellfish Immunol,* 23**,** 378-89.

KROEMER, G. & REED, J. C. (2000) Mitochondrial control of cell death. *Nat Med,* 6**,** 513-9.

KROSSOY, B., DEVOLD, M., SANDERS, L., KNAPPSKOG, P. M., ASPEHAUG, V., FALK, K., NYLUND, A., KOUMANS, S., ENDRESEN, C. & BIERING, E. (2001) Cloning and identification of the infectious salmon anaemia virus haemagglutinin. *J Gen Virol,* 82**,** 1757-65.

KROSSOY, B., HORDVIK, I., NILSEN, F., NYLUND, A. & ENDRESEN, C. (1999) The putative polymerase sequence of infectious salmon anemia virus suggests a new genus within the Orthomyxoviridae. *J Virol,* 73**,** 2136-42.

KUUSISTO, E., SUURONEN, T. & SALMINEN, A. (2001) Ubiquitin-binding protein p62 expression is induced during apoptosis and proteasomal inhibition in neuronal cells. *Biochem Biophys Res Commun,* 280**,** 223-8.

LENSCHOW, D. J., LAI, C., FRIAS-STAHELI, N., GIANNAKOPOULOS, N. V., LUTZ, A., WOLFF, T., OSIAK, A., LEVINE, B., SCHMIDT, R. E., GARCIA-SASTRE, A., LEIB, D. A., PEKOSZ, A., KNOBELOCH, K. P., HORAK, I. & VIRGIN, H. W. T. (2007) IFN-stimulated gene 15 functions as a critical antiviral molecule against influenza, herpes, and Sindbis viruses. *Proc Natl Acad Sci U S A,* 104**,** 1371-6.

LIVAK, K. J. & SCHMITTGEN, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods,* 25**,** 402-8.

MARUOKA, S., HASHIMOTO, S., GON, Y., NISHITOH, H., TAKESHITA, I., ASAI, Y., MIZUMURA, K., SHIMIZU, K., ICHIJO, H. & HORIE, T. (2003) ASK1 regulates influenza virus infection-induced apoptotic cell death. *Biochem Biophys Res Commun,* 307**,** 870-6.

MATEJUSOVA, I., FELIX, B., SORSA-LESLIE, T., GILBEY, J., NOBLE, L. R., JONES, C. S. & CUNNINGHAM, C. O. (2006) Gene expression profiles of some immune relevant genes from skin of susceptible and responding Atlantic salmon (Salmo salar L.) infected with Gyrodactylus salaris (Monogenea) revealed by suppressive subtractive hybridisation. *Int J Parasitol,* 36**,** 1175-83.

MCBEATH, A. J., COLLET, B., PALEY, R., DURAFFOUR, S., ASPEHAUG, V., BIERING, E., SECOMBES, C. J. & SNOW, M. (2006) Identification of an interferon antagonist protein encoded by segment 7 of infectious salmon anaemia virus. *Virus Res,* 115**,** 176-84.

MCBEATH, A. J., SNOW, M., SECOMBES, C. J., ELLIS, A. E. & COLLET, B. (2007) Expression kinetics of interferon and interferon-induced genes in Atlantic salmon (Salmo salar) following infection with infectious pancreatic necrosis virus and infectious salmon anaemia virus. *Fish Shellfish Immunol,* 22**,** 230-41.

MIKALSEN, A. B., TEIG, A., HELLEMAN, A. L., MJAALAND, S. & RIMSTAD, E. (2001) Detection of infectious salmon anaemia virus (ISAV) by RT-PCR after cohabitant exposure in Atlantic salmon Salmo salar. *Dis Aquat Organ,* 47**,** 175-81.

MJAALAND, S., RIMSTAD, E., FALK, K. & DANNEVIG, B. H. (1997) Genomic characterization of the virus causing infectious salmon anemia in Atlantic salmon (Salmo salar L.): an orthomyxo-like virus in a teleost. *Journal of Virology,* 71**,** 7681-6.

MOMOSE, F., NAITO, T., YANO, K., SUGIMOTO, S., MORIKAWA, Y. & NAGATA, K. (2002) Identification of Hsp90 as a stimulatory host factor involved in influenza virus RNA synthesis. *J Biol Chem,* 277**,** 45306-14.

MOULDING, D. A., QUAYLE, J. A., HART, C. A. & EDWARDS, S. W. (1998) Mcl-1 expression in human neutrophils: regulation by cytokines and correlation with cell survival. *Blood,* 92**,** 2495-502.

MULLAUER, L. (2006) Apotosis; Regulatory Genes and Disease. IN SONS, J. W. (Ed.) *ENCYCLOPEDIA OF LIFE SCIENCES.*

MULTHOFF, G. (2007) Heat shock protein 70 (Hsp70): Membrane location, export and immunological relevance. *Methods,* 43**,** 229-37.

MUNIR, K. & KIBENGE, F. S. (2004) Detection of infectious salmon anaemia virus by real-time RT-PCR. *J Virol Methods,* 117**,** 37-47.

NEUMANN, G., KAWAOKA,Y (2007) Influenza Viruses. IN JOHN WILEY & SONS, L. (Ed.) *ENCYCLOPEDIA OF LIFE SCIENCES.*

NIESTERS, H. G. (2001) Quantitation of viral load using real-time amplification techniques. *Methods,* 25**,** 419-29.

NIETO-MIGUEL, T., GAJATE, C., GONZALEZ-CAMACHO, F. & MOLLINEDO, F. (2007) Proapoptotic role of Hsp90 by its interaction with c-Jun N-terminal kinase in lipid rafts in edelfosine-mediated antileukemic therapy. *Oncogene.*

NIJHAWAN, D., FANG, M., TRAER, E., ZHONG, Q., GAO, W., DU, F. & WANG, X. (2003) Elimination of Mcl-1 is required for the initiation of apoptosis following ultraviolet irradiation. *Genes Dev,* 17**,** 1475-86.

OLSEN, C. W., KEHREN, J. C., DYBDAHL-SISSOKO, N. R. & HINSHAW, V. S. (1996) bcl-2 alters influenza virus yield, spread, and hemagglutinin glycosylation. *J Virol,* 70**,** 663-6.

OYAMA, M., MYOKAI, F., OHIRA, T., SHIOMI, N., YAMASHIRO, K., ARAI, H., NISHIMURA, F. & TAKASHIBA, S. (2005) Isolation and expression of FIP-2 in wounded pulp of the rat. *J Dent Res,* 84**,** 842-7.

PADDOCK, S. (2001) Confocal Laser Scanning Light Microscopy. IN JOHN WILEY & SONS, L. (Ed.) *ENCYCLOPEDIA OF LIFE SCIENCES.*

PANCHUK-VOLOSHINA, N., HAUGLAND, R. P., BISHOP-STEWART, J., BHALGAT, M. K., MILLARD, P. J., MAO, F., LEUNG, W. Y. & HAUGLAND, R. P. (1999) Alexa dyes, a series of new fluorescent dyes that yield exceptionally bright, photostable conjugates. *J Histochem Cytochem,* 47**,** 1179-88.

PAPASAVVAS, E., SUN, J., LUO, Q., MOORE, E. C., THIEL, B., MACGREGOR, R. R., MINTY, A., MOUNZER, K., KOSTMAN, J. R. & MONTANER, L. J. (2005) IL-13 acutely augments HIV-specific and recall responses from HIV-1-infected subjects in vitro by modulating monocytes. *J Immunol,* 175**,** 5532-40.

PARHAM, P. (2005) *The Immune System*, Garland Sciences.

PFAFFL, M. W., HORGAN, G. W. & DEMPFLE, L. (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res,* 30**,** e36.

PIRHONEN, J., SARENEVA, T., KURIMOTO, M., JULKUNEN, I. & MATIKAINEN, S. (1999) Virus infection activates IL-1 beta and IL-18 production in human macrophages by a caspase-1-dependent pathway. *J Immunol,* 162**,** 7322-9.

PURCELL, M. K., KURATH, G., GARVER, K. A., HERWIG, R. P. & WINTON, J. R. (2004) Quantitative expression profiling of immune response genes in rainbow trout following infectious haematopoietic necrosis virus (IHNV) infection or DNA vaccination. *Fish Shellfish Immunol,* 17**,** 447-62.

RESTIFO, N. P. (2000) Building better vaccines: how apoptotic cell death can induce inflammation and activate innate and adaptive immunity. *Curr Opin Immunol,* 12**,** 597-603.

RIMSTAD, E., MJAALAND, S., SNOW, M., MIKALSEN, A. B. & CUNNINGHAM, C. O. (2001) Characterization of the infectious salmon anemia virus genomic segment that encodes the putative hemagglutinin. *J Virol,* 75**,** 5352-6.

RIRIE, K. M., RASMUSSEN, R. P. & WITTWER, C. T. (1997) Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Anal Biochem,* 245**,** 154-60.

RITCHIE, R. J., HEPPELL, J., COOK, M. B., JONES, S. & GRIFFITHS, S. G. (2001) Identification and characterization of segments 3 and 4 of the ISAV genome. *Virus Genes,* 22**,** 289-97.

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

ROKENES, T. P., LARSEN, R. & ROBERTSEN, B. (2007) Atlantic salmon ISG15: Expression and conjugation to cellular proteins in response to interferon, double-stranded RNA and virus infections. *Mol Immunol,* 44**,** 950-9.

ROLLAND, J. B., BOUCHARD, D., COLL, J. & WINTON, J. R. (2005) Combined use of the ASK and SHK-1 cell lines to enhance the detection of infectious salmon anemia virus. *J Vet Diagn Invest,* 17**,** 151-7.

SAVAN, R., KONO, T., IGAWA, D. & SAKAI, M. (2005) A novel tumor necrosis factor (TNF) gene present in tandem with theTNF-alpha gene on the same chromosome in teleosts. *Immunogenetics,* 57**,** 140-50.

SCHULTZ-CHERRY, S., DYBDAHL-SISSOKO, N., NEUMANN, G., KAWAOKA, Y. & HINSHAW, V. S. (2001) Influenza virus ns1 protein induces apoptosis in cultured cells. *J Virol,* 75**,** 7875-81.

SCHULTZ-CHERRY, S. & HINSHAW, V. S. (1996) Influenza virus neuraminidase activates latent transforming growth factor beta. *J Virol,* 70**,** 8624-9.

SELBY, P. L., DAVIES, M. & MEE, A. P. (2006) Canine distemper virus induces human osteoclastogenesis through NF-kappaB and sequestosome 1/P62 activation. *J Bone Miner Res,* 21**,** 1750-6.

SNOW, M. & CUNNINGHAM, C. O. (2001) Characterisation of the putative nucleoprotein gene of infectious salmon anaemia virus (ISAV). *Virus Res,* 74**,** 111-8.

SNOW, M., RAYNARD, R. S., MURRAY, A. G., BRUNO, D. W., KING, J. A., GRANT, R., BRICKNELL, I. R., BAIN, N. & GREGORY, A. (2003a) An evaluation of current diagnostic tests for the detection of infectious salmon anaemia virus (ISAV) following experimental water-borne infection of Atlantic salmon, Salmo salar L. *Journal of Fish Diseases,* 26**,** 135-45.

SNOW, M., RITCHIE, R., ARNAUD, O., VILLOING, S., ASPEHAUG, V. & CUNNINGHAM, C. O. (2003b) Isolation and characterisation of segment 1 of the infectious salmon anaemia virus genome. *Virus Res,* 92**,** 99-105.

STOTT, D. I. (1989) Immunoblotting and dot blotting. *J Immunol Methods,* 119**,** 153-87.

TAFALLA, C., COLL, J. & SECOMBES, C. J. (2005) Expression of genes related to the early immune response in rainbow trout (Oncorhynchus mykiss) after viral haemorrhagic septicemia virus (VHSV) infection. *Dev Comp Immunol,* 29**,** 615-26.

TAKIZAWA, T., OHASHI, K. & NAKANISHI, Y. (1996) Possible involvement of double-stranded RNA-activated protein kinase in cell death by influenza virus infection. *J Virol,* 70**,** 8128-32.

TAKLE, H., MCLEOD, A. & ANDERSEN, O. (2006) Cloning and characterization of the executioner caspases 3, 6, 7 and Hsp70 in hyperthermic Atlantic salmon (Salmo salar) embryos. *Comp Biochem Physiol B Biochem Mol Biol,* 144**,** 188-98.

THOMPSON, C. B. (1995) Apoptosis in the pathogenesis and treatment of disease. *Science,* 267**,** 1456-62.

THOMSON, B. J. (2001) Viruses and apoptosis. *Int J Exp Pathol,* 82**,** 65-76.

THORUD, K., AND DJUPVIK, H (1988) Infectious anemia in Atlantic salmon (*Salmo salar L.*). *Bull. Eur Assoc. Fish Pathol.,* 8**,** 109-111.

TURPIN, E., LUKE, K., JONES, J., TUMPEY, T., KONAN, K. & SCHULTZ-CHERRY, S. (2005) Influenza virus infection increases p53 activity: role of p53 in cell death and viral replication. *J Virol,* 79**,** 8802-11.

VAN DELFT, M. F. & HUANG, D. C. (2006) How the Bcl-2 family of proteins interact to regulate apoptosis. *Cell Res,* 16**,** 203-13.

VOGELSTEIN, B., LANE, D. & LEVINE, A. J. (2000) Surfing the p53 network. *Nature,* 408**,** 307-10.

WALLIN, R. P., LUNDQVIST, A., MORE, S. H., VON BONIN, A., KIESSLING, R. & LJUNGGREN, H. G. (2002) Heat-shock proteins as activators of the innate immune system. *Trends Immunol,* 23**,** 130-5.

WESSENDORF, M. W. & BRELJE, T. C. (1992) Which fluorophore is brightest? A comparison of the staining obtained using fluorescein, tetramethylrhodamine, lissamine rhodamine, Texas red, and cyanine 3.18. *Histochemistry,* 98**,** 81-5.

WURZER, W. J., EHRHARDT, C., PLESCHKA, S., BERBERICH-SIEBELT, F., WOLFF, T., WALCZAK, H., PLANZ, O. & LUDWIG, S. (2004) NF-kappaB-dependent induction of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas/FasL is crucial for efficient influenza virus propagation. *J Biol Chem,* 279**,** 30931-7.

WURZER, W. J., PLANZ, O., EHRHARDT, C., GINER, M., SILBERZAHN, T., PLESCHKA, S. & LUDWIG, S. (2003) Caspase 3 activation is essential for efficient influenza virus propagation. *Embo J,* 22**,** 2717-28.

YANG, R. Y. & LIU, F. T. (2003) Galectins in cell growth and apoptosis. *Cell Mol Life Sci,* 60**,** 267-76.

ZAGON, I. S., SASSANI, J. W. & MCLAUGHLIN, P. J. (1998) Re-epithelialization of the rabbit cornea is regulated by opioid growth factor. *Brain Res,* 803**,** 61-8.

ZAGON, I. S., VERDERAME, M. F. & MCLAUGHLIN, P. J. (2002) The biology of the opioid growth factor receptor (OGFr). *Brain Res Brain Res Rev,* 38**,** 351-76.

ZAGON, I. S., WU, Y. & MCLAUGHLIN, P. J. (1994) Opioid growth factor inhibits DNA synthesis in mouse tongue epithelium in a circadian rhythm-dependent manner. *Am J Physiol,* 267**,** R645-52.

ZAGON, I. S., WU, Y. & MCLAUGHLIN, P. J. (1996) The opioid growth factor, [Met5]-enkephalin, and the zeta opioid receptor are present in human and mouse skin and tonically act to inhibit DNA synthesis in the epidermis. *J Invest Dermatol,* 106**,** 490-7.

ZHIRNOV, O. P., KONAKOVA, T. E., WOLFF, T. & KLENK, H. D. (2002a) NS1 protein of influenza A virus down-regulates apoptosis. *J Virol,* 76**,** 1617-25.

ZHIRNOV, O. P., KSENOFONTOV, A. L., KUZMINA, S. G. & KLENK, H. D. (2002b) Interaction of influenza A virus M1 matrix protein with caspases. *Biochemistry (Mosc),* 67**,** 534-9.