

Protein-protein interactions involved in the interferon
antagonistic activity of infectious salmon anemia virus
(ISAV).

Samuel N.A. Fredriksson

NORWEGIAN UNIVERSITY OF LIFE SCIENCES
DEPARTMENT OF CHEMISTRY, BIOTECHNOLOGY AND FOOD SCIENCE
MASTER THESIS 60 CREDITS 2011



Acknowledgements

This master thesis project was carried out at the Norwegian National Veterinary Institute (NVI) with examination at the Norwegian University of Life Sciences (UMB). The research was financed by funding from the Research Council of Norway, and carried out between the 1st of September 2010 and the 15th of May 2011.

First of all, I wish to thank my external supervisor PhD Siri Mjaaland, Senior Scientist (NVI), for her inspiring research, great ambitions, enthusiasm and constructive guidance, and for including me as a part of her research group. I would also like to thank my internal supervisor Professor Helge Holo (UMB). Special thanks goes out to PhD post.docs Jon Ramsell and Helena Hauge, both at the NVI, for patiently sharing their knowledge and for great support at the lab. The research team of Professor Jorunn Jørgensen at the University of Tromsø (UIT), has contributed with experimental advice, vectors and a memorable visit in Tromsø during the RCN Fish Health Conference in January 2011.

Finally, for believing in me and supporting me, my family, Henriette Hustadnes Hagen and Liam Fredriksson.

Oslo, May 12th 2011

Samuel Fredriksson

Abstract

Infectious salmon anemia virus (ISAV) is an aquatic influenza-like virus that infects Atlantic salmon, causing high mortalities, and with huge economical impact in the fish farming industry. Outbreaks of new virulent strains is an emerging threat to the aquaculture industry as no appropriate vaccines to trigger the acquired immune response are yet developed. The interaction between the infectious agent and its host is here investigated focusing on the innate immune system of the Atlantic salmon (*Salmo Salar*) and their interactions with ISAV. In the host's innate response, the type 1 interferon (IFN) stands out as the first line of defence upon viral infection. ISAV, as other genera of *Orthomyxoviridae*, has evolved multiple ways to avoid the immediate antiviral effects of the IFN. In ISAV the segments 7ORF1 and 8ORF2 have been shown to exert interferon antagonistic activity, but the detailed mechanisms behind this phenomena are yet unknown.

In this project, the IFN antagonistic activity of ISAV has been studied. By studying the protein-protein interactions, the understanding of how the immune system is suppressed by ISAV during an ISAV infection can be increased. The effect the ISAV proteins have on the IFN system proteins of the signalling pathway from virus recognition to the interferon regulatory factors (IRFs) were analysed. This was enabled using the method of Yeast-2-Hybrid screening for interactions and using co-immunoprecipitation for verification of the interactions. We found the interactions between IRF3 and the protein encoded from ISAV segment 8ORF2 that have similarities with the IFN antagonistic ML-protein of Thogoviruses interacting with the IRF3 located in the nucleus. The other stated IFN antagonist of ISAV, segment 7ORF1, located in the cytoplasm was identified with interactions with IRF7a. It is discussed whether this interaction has features in common with the Influenza A NS segment that interact with IRF3 in mammals. For further verification of the protein-protein interactions, the ISAV segment 8ORF2 interaction with IRF3 were analysed by co-immunoprecipitation, and a weak interaction was verified. It is discussed whether the weak interaction reflects the low transfection efficiency of IRF3.

Keywords: Orthomyxoviruses; Infectious salmon anemia virus; ISAV; interferon antagonism; protein-protein interactions; virus-host interactions

Sammendrag

Infeksiøs lakseanemi virus (ILAV) er et akvatisk influensa-lignende virus som infiserer Atlantisk laks og forårsaker stor dødlighet. Til nå er det ikke utviklet effektive vaksiner som gir god beskyttelse mot ILAV. Utbrudd av nye virulente stammer utgjør derfor en voksende trussel mot fiskeindustrien. Samspillet mellom smittestoffet og dens vert er i denne studien undersøkt med fokus på interaksjonene mellom det medfødte immunsystemet hos atlantisk laks (*Salmo Salar*) og ILAV. I vertens medfødte immunrespons utgjør type I interferon (IFN) et førstelinjeforsvaret ved virale infeksjoner. ILAV har som andre genera av *Orthomyxoviridae* utviklet flere måter å unnslipe den umiddelbare antivirale effekten av IFN. ILA virus segmentene 7ORF1 og 8ORF2 har begge IFN antagonistisk aktivitet, men de underliggende mekanismerne bak dette er fortsatt ukjente.

I denne studien har de IFN antagonistiske aktivitetene hos ILAV blitt studert på basis av protein-protein interaksjoner for å øke forståelse av hvordan ILAV unnsliper vertens immunforsvar i løpet av en infeksjon. Vi har analysert IFN system proteiner i signalkjeden fra virusdeteksjon til interferon regulatoriske faktorer (IRF)s sammen med proteiner av ILAV. Dette ble gjort ved bruk av gjær-2-hybrid for å screene protein-protein interaksjoner, Co-immunprecipitering ble så benyttet for å verifisere funnene. Ved screening ble det funnet interaksjoner mellom IRF3 og ILAV segment 8ORF2, begge proteinene er lokalisert i kjernen. Om denne interaksjonen kan ha de samme funksjonene som de IFN antagonistiske ML protein av Thogovirus blir diskutert, begge er lokalisert i kjernen. Det ble også identifisert en interaksjon mellom segment 7ORF1 IRF7a. Det blir drøftet om denne interaksjonen har trekk til felles med den IFN antagonistiske interaksjonen NS 1 segmentet til Influenza A har med IRF3 i pattedyr begge proteiner er lokalisert i cytoplasma. En svak protein-protein interaksjon mellom ISAV segment 8ORF2 og IRF3 ble verifisert med co-immunpresipitering. Det blir diskutert om den svake interaksjonen reflekterer den lave transkripsjons effektiviteten av IRF3 i vert.

Nøkkelord: Orthomyxovirus; infektøs lakseanemi virus; ILAV; interferon antagonisme; protein-protein interaksjoner; virus-vert-interaksjoner

Table of Contents

CHAPTER 1. INTRODUCTION	12
1.1 INFECTIOUS SALMON ANEMIA (ISA).....	12
1.2 INFECTIOUS SALMON ANEMIA VIRUS (ISAV).....	12
1.2.1 ISAV segments.....	13
1.2.2 ISAV replication cycle.....	15
1.3 THE VIRAL DEFENCE OF ATLANTIC SALMON.....	16
1.3.1 <i>The acquired immune response in Atlantic salmon upon viral infection and vaccination</i>	18
1.4 RECOGNITION OF VIRAL RNA AND SIGNALLING PATHWAYS.....	19
1.5 OTHER ORTHOMYXOVIRUSES WITH IFN-ANTAGONISTIC GENES.....	20
1.6 COILED-COIL INTERACTIONS.....	21
1.7 AIM OF STUDY.....	23
CHAPTER 2. KEY MOLECULAR TECHNIQUES	24
2.1 YEAST-TWO-HYBRID (Y2H).....	24
2.2 CO-IMMUNOPRECIPITATION (CO-IP).....	26
CHAPTER 3 MATERIALS	28
3.1 CHEMICALS.....	28
3.2 KITS AND ENZYMES.....	29
3.3 VECTORS, PRIMERS.....	29
3.4 SOLUTIONS.....	30
3.5 BIOLOGICAL MATERIAL.....	31
3.6 LABORATORY EQUIPMENT.....	33
3.7 SOFTWARE.....	34
CHAPTER 4. METHODS	35
4.1 GATEWAY CLONING OF ATLANTIC SALMON AND ISAV GENES.....	35
4.1.1 <i>Expression vectors</i>	35
4.1.2 <i>Primer design for Gateway cloning</i>	35
4.1.3 <i>Polymerase chain reaction (PCR) for the pENTR vector</i>	36
4.1.4 <i>Cloning into pENTR D-TOPO vector (Entry clone)</i>	39
4.2. SCREENING FOR PROTEIN-PROTEIN INTERACTIONS USING YEAST TWO HYBRID (Y2H).....	42
4.2.1 <i>Expression vectors for Yeast-two-hybrid</i>	42
4.2.2 <i>Sequencing of the expression vectors for Yeast-Two-Hybrid</i>	45
4.2.3 <i>Protein expression analysis of Y2H-expression vectors by Western Blotting</i>	45
4.2.4 <i>Mating of the pGBD vector in PJ69-2A cells and pGAD vector in Y187 cells</i>	47
4.2.5 <i>Negative and positive control for Y2H</i>	48
4.3. CO-IMMUNOPRECIPITATION (CO-IP).....	48
4.3.1 <i>Expression vectors for co-Immune precipitation</i>	48
4.3.2 <i>Sequencing of the expression vectors for co-immunoprecipitation</i>	48
4.3.3 <i>Positive and negative controls for Co-IP</i>	49
4.3.4 <i>Transfection of HEK 293T cells for IP</i>	49
4.3.5 <i>Transfection control</i>	50
4.3.6 <i>Antibody binding to Dynabeads</i>	51
4.3.7 <i>Precipitation</i>	51
4.3.8 <i>Negative and positive control for co-IP</i>	52
4.3.9 <i>Western Blotting for Co-IP</i>	52
4.4 BIOINFORMATICS.....	53
4.4.1 <i>Comparison of Y2H and Co-IP vectors with genes from GenBank</i>	53

4.4.2	<i>Coiled-coil prediction</i>	53
4.4.3	<i>Alignment of IRF3 from Human and Atlantic salmon</i>	53
CHAPTER 5.	RESULTS	54
5.1	SCREENING FOR PROTEIN-PROTEIN INTERACTIONS USING YEAST-TWO-HYBRID (Y2H)	54
5.1.1	<i>Identifying the vector inserts used in Y2H experiments</i>	54
5.1.2	<i>Western blot for Y2H</i>	56
5.1.3	<i>Yeast-Two-Hybrid mating</i>	58
5.2	VERIFICATION OF PROTEIN-PROTEIN INTERACTIONS USING CO-IMMUNOPRECIPITATION	60
5.2.1	<i>Identification of the pDEST –GFP vector sequence used in co-immunoprecipitation</i>	60
5.2.2	<i>Transfection control by detection of fluorescence from translated pDEST-GFP vectors</i>	61
5.2.3	<i>Detection of Co-precipitated proteins on Western Blot</i>	62
5.3	FUNCTIONAL STUDIES.....	65
5.3.1	<i>Coiled-coil prediction</i>	65
5.3.2	<i>Conserved regions within the IRF genes</i>	66
CHAPTER 6.	DISCUSSION	67
6.1	SCREENING FOR PROTEIN-PROTEIN INTERACTIONS WITH Y2H.....	67
6.2	VERIFICATION OF PROTEIN-PROTEIN INTERACTION USING CO- IMMUNOPRECIPITATION (CO-IP).....	69
6.3	PROTEIN-PROTEIN INTERACTIONS AND THEIR PUTATIVE FUNCTION IN THE IMMUNE SYSTEM.	70
6.4	FURTHER ASPECTS.....	72
CHAPTER 7.	LITERATURE	74
Appendix 1	ISAV segment molecular size measured with polyclonal antibodies	
Appendix 2	Sequneces of expression vectors for Yeast-two-hybrid	

Abbreviations

NVI: National Veterinary Institute

UIT: University of Tromsø

ISA: Infectious salmon anemia

ISAV: Infectious salmon anemia virus

ISAV 4: Isolate of ISAV from the Glesvær outbreak in 1990

INV: Influenza virus

(ss) RNA: Single stranded RNA

PB: Basic polymerase

PA: Acid polymerase

NP: Nucleic protein

F: Fusion protein

HE: Hemagglutinin-Esterase

ORF: Open reading frame

vRNA: Viral RNA

cRNA: Complementary RNA

RNP: ribonucleic protein

PRR: Pattern Recognition Receptors

IFN: Interferon

IRF: IFN regulatory factors

NF- κ B: Nuclear factor

TLR: Toll like receptor

MHC: Major histocompatibility complex

APC: Antigen presenting cell

Y2H: Yeast-two-hybrid

Leu: Leucine

Ade: Adenine

Trp: Tryptophane

His: Histidine

Co-IP: Co-Immunoprecipitation

HEK 293T: Human embryo kidney 293T cells

Chapter 1. Introduction

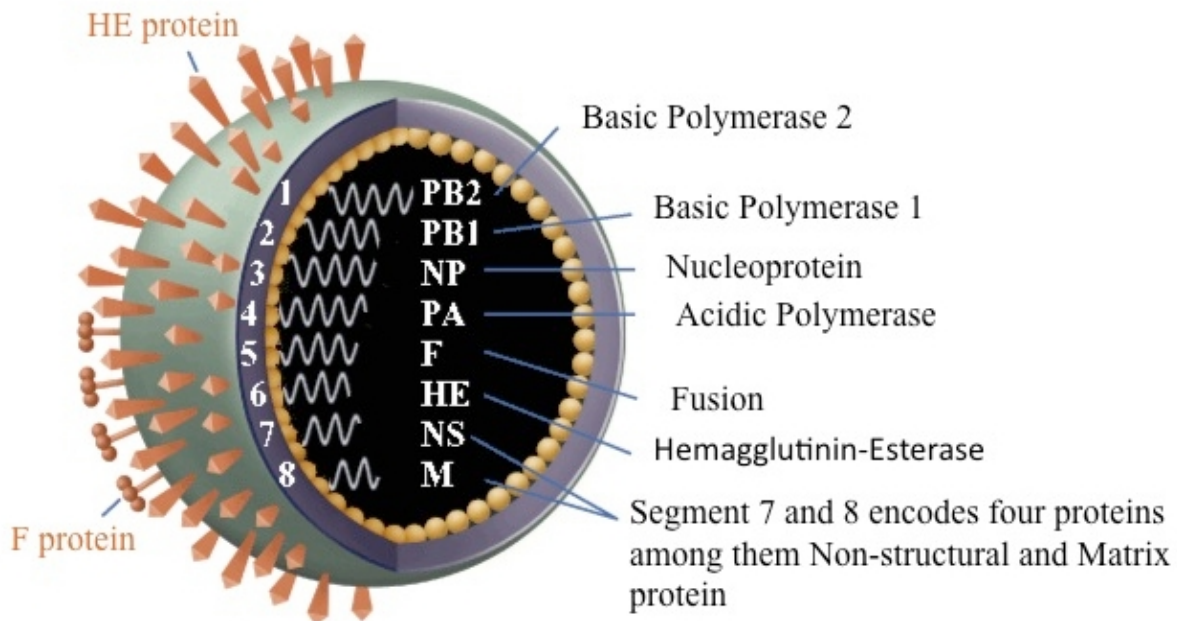
1.1 *Infectious salmon anemia (ISA)*

Since the early 1980s the aqua culture of the Atlantic salmon (*Salmo salar*) has developed into a huge industry. In 2007 the production in Norway reached 800 000 tons and made up for 44% of the global production (Solar, 2009) which had a value of 2.9 billion US\$. Infectious salmon anemia (ISA) is a disease that counts as an emerging threat to the salmon farming industry. The disease was first confirmed 1984 in Norway and epidemic outbreaks of ISA increased in the late 1980s and the early 1990s (Vågsholm, et al., 1994). The mortality rate during an outbreak of ISA has reached 50% in marine farms (Thorud & Djupvik, 1988) and 90% during *in vitro* trials (Hetland, et al., 2010). The preventive measures conducted by the European Commission forces the farmer to eradication of the entire fish stock and waste once ISA is detected, causing huge economic losses for the farmer. Clinical signs of ISA are anemia that arises from the infected and damaged blood cells, damages to the walls of the blood vessels, often in combination with pale gills and a swollen liver. During the last decade the disease has spread to the Faroe Islands, Scotland, Canada, USA, and recently an outbreak was detected in fish farms in Chile (Kibenge, et al., 2009) . Since the first epidemic outbreak of ISAV in Norway, the research effort has been strengthened to prevent new outbreaks.

1.2 *Infectious salmon anemia virus (ISAV)*

Infectious salmon anemia virus (ISAV) causing infectious salmon anemia (ISA) was first isolated in 1995 (Dannevig, et al., 1995), and characterized to an aquatic orthomyxovirus in 1997 (Mjaaland, et al., 1997). Orthomyxoviruses, like Influenza A (INVA), B (INVB), C (INVC), Thogovirus and the current studied ISA virus genera, have characteristics of negative-sense, single-stranded (ss) RNA and segmented RNA genome (Knipe & Howley, 2007). The INV A, B and C have the ability to evolve their virulence through genetic reassortment within the segmented RNA genome (Palese, 1977) and ISAV has been shown to

evolve virulence through the same mechanism (Markussen, et al., 2008). The ISAV particle has eight genomic segments encoding for at least 10 viral proteins (Biering, et al., 2002). Segment one is the largest and segment eight is the smallest, see figure 1.



Modified from Wuehrich, B. 2003

Figure 1. Viral segments and their encoding viral proteins of ISAV.

The viral segments 1, 2 and 4 encode the viral polymerases PB2, PB1 and PA, respectively, while the viral segment 3 encode the nucleoprotein (NP). Gene segment 5 and 6 encode the surface fusion (F) protein and Hemagglutinin-esterase (HE) protein. The two smallest segments (segment 7 and 8) encode for two proteins each.

1.2.1 ISAV segments

To understand how the virus infection triggers the host immune response we need to look deeper into each virus protein and their interactions with key proteins of the salmon immune system.

Viral Polymerases of ISAV

The RNA dependent RNA polymerases have a merged structure of the three proteins; polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), and polymerase acidic protein (PA). The basic polymerase PB2 introduces the cap binding sites and as with the

Influenza viruses all ISAV segments are capped with host “look-a like” structures. This enables capped viral mRNA to leave the core for translation in the cytoplasm (Snow, et al., 2003). The basic polymerase PB1 act as a backbone complex during vRNA production (Snow et al., 2003), while the acid polymerase PA is most likely necessary for both replication and transcription of vRNA (Falk, et al., 2004).

Nucleoprotein (NP)

The Nucleoprotein (NP) of ISAV is suggested to have the same configuration in the lumen as Influenza viruses. That form ribonucleic-protein complexes (RNPs) through binding of the viral RNA (vRNA) to the viral polymerases PB2, PB1, PA and the NP (Falk, et al., 2004). RNPs synthesize complementary RNA (cRNA) as template for the synthesis of new vRNA. Finally, the nucleoprotein also has a nuclear localisation protein (NLS) transport signal peptide that ensures vRNA transport into the nucleus (Asphaug, et al., 2004).

Fusion (F)

The first surface protein of ISAV segment 5 is responsible for fusion of viral and cellular membranes (Aspehaug, et al., 2005). All enveloped virus which have multi-layered shells and one lipid enveloped surface needs the fusion protein to merge the viral and host cell membranes in order for the virus to entry the host cell where the virus replicates (Harrison, 2005).

Hemagglutinin-esterase (HE)

Segment 6 encodes the second surface protein handling the host-cell receptor-binding and -release activity (Aspehaug, et al., 2005). First, the virus binds to the cell surface using acetylated sialic acid as a receptor on the cell surface (Hellebo, et al., 2004). Second, the esterase properties is involved in the releases of ISAV from the cell (Aspehaug, et al., 2005).

Proteins encoded from ISAV segment 7 and 8

The two smallest ISAV fragments 7 and 8 encodes at least four proteins, where two of them have type-1 interferon antagonistic activity; 7ORF1 (McBeath et al., 2006) and 8ORF2 (García-Rosado et al., 2008). Both have been located in the host by confocal microscopy;

7ORF1 to the cytoplasm and the 8ORF2 to the nucleus (García-Rosado et al., 2008). Segment 7ORF1 encodes a non-structural protein expressed early in infection, while the 8ORF2 segment encodes a structural protein expressed late in infection (Biering, et al., 2002). The ISAV 7ORF2 is a possible analogue to the INVs nuclear export proteins (NEP) that export RNPs from the nucleus to the cytoplasm (O'Neill, et al., 1998). Segment 8ORF1 encodes the matrix protein located under the lipid bilayer (Falk, et al., 2004).

1.2.2 ISAV replication cycle

The ISAV replication cycle is initiated by binding of the HE protein to acetylated sialic acid specific receptors on the cell surface, which triggers a receptor mediated endocytosis (Knipe & Howley, 2007). The adsorption of ISAV to the cell surface results in inward budding of the plasma membrane of the cell and low pH in the endocytic compartments triggers fusion of the viral and endosome membranes. Resulting in the uncoating of ISAV and release of vRNA into the cytoplasm (Skehel & Wiley, 2000). The NLS in the nucleoprotein then ensures the transport of vRNA into the nucleus (Asphaug, et al., 2004) where the virus segments uses the cells own expression machinery. The viral genome (vRNA) is replicated from vRNA through complementary RNA (cRNA) to get positive-sense translating mRNA. In other words vRNA is the template for both mRNA and cRNA (Knipe & Howley, 2007). Early translated viral proteins necessary for secondary mRNA transcription, cRNA- and vRNA synthesis are translated in the cytoplasm and re-imported as proteins to the nucleus, as illustrated in figure 2.

Synthesised vRNPs and viral mRNA are exported from the nucleus by the nuclear export proteins (NEP) to the cytoplasm. The viral mRNA is translated in the cytoplasm and the posttranslational processing of HE and F proteins delivers these viral membrane proteins on the surface of the host cell. The M proteins along with the eight vRNPs assemble close to the HE and F coated plasma membrane, where the RNA genome is packed and buds off, coated with the lipid membrane derived from the host cell, see figure 2. The M protein, mediates the budding of a new ISAV particle, and the closure of buds and the virion release is mediated by the esterase of the HE-esterase (Aspehaug, et al., 2005). The whole replication cycle is illustrated in figure 2.

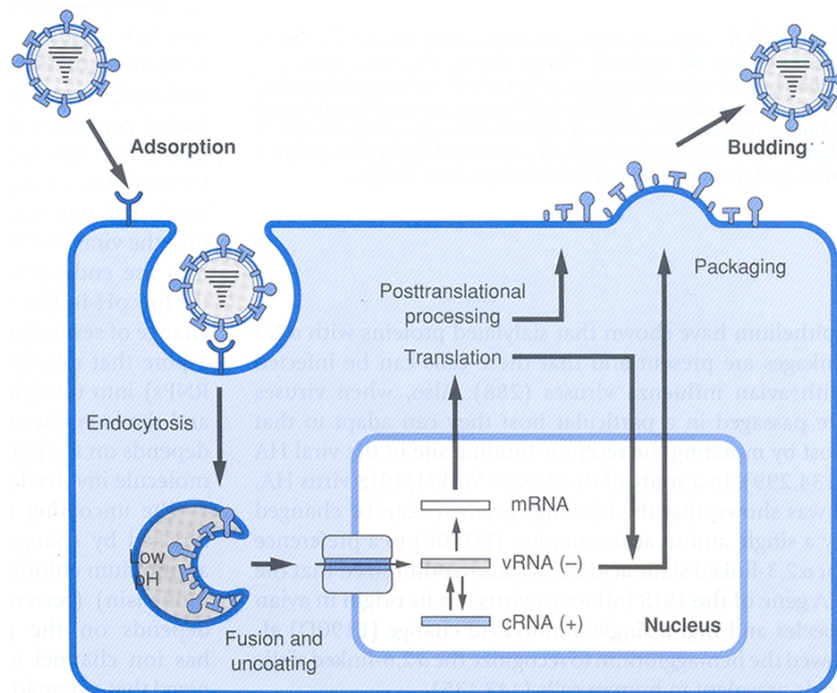


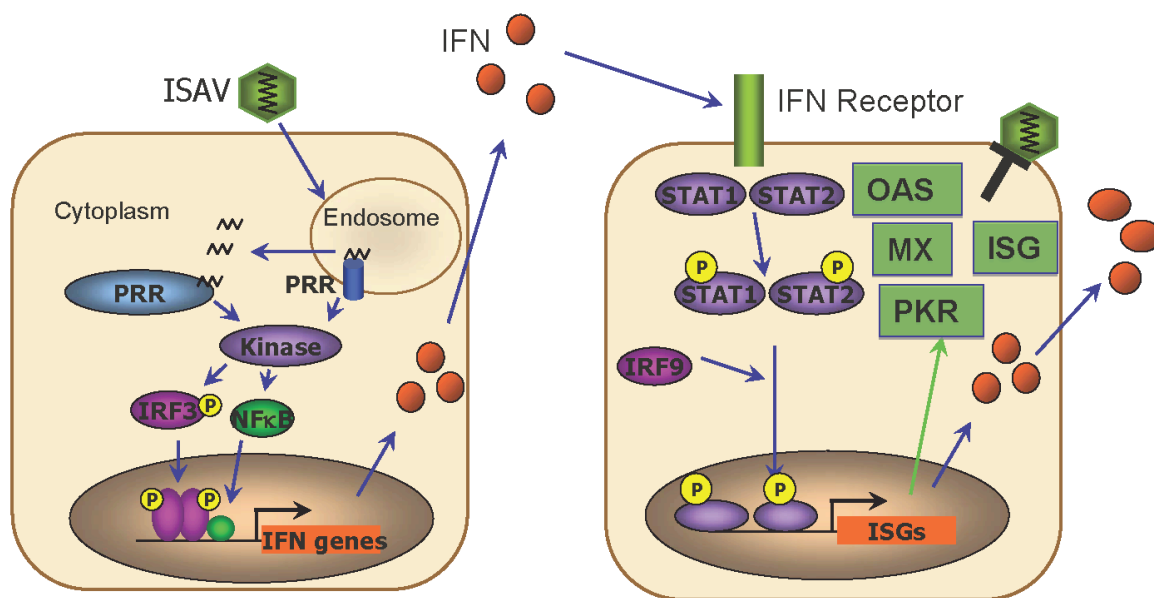
Figure 2. Illustration of the Influenza virus replication (Knipe, 2007).

1.3 The Viral defence of Atlantic salmon

A virus is an infectious, intracellular parasitic particle dependent on the host cell to implement replication. The host's first line of defence against viral infection is the innate immunity that acts rapidly with minor specificity but putting the viral genome under strong selection pressure forcing evolutionary adaptation for viral replication. After the first line of defence the innate response precedes the acquired immunity that act after several days or weeks (Samuel, 2001) with high specificity and often provides lifelong immunity. However, the Atlantic salmon lacks bone marrow and defined lymphoid tissue. Instead, the head kidney serves as major lymphoid organ in addition to the thymus and spleen (Press & Evensen,1999). Many of the proteins involved in the salmon immunity have recently been characterized with the same properties as for mammals. The cytokine interferon has also been characterized in Atlantic salmon (Kileng, et al, 2009). In fact the interferon has a highly central role in the innate immune response upon viral infection (Garcia-Sastre & Biron, 2006).

Interferon Type I system

The Interferon (IFN) was discovered by Isacs and Lindenmann (1957) and described as an agent that inhibited the replication of influenza virus. IFN is today used therapeutically in treatment of some viral diseases (Borden, et al., 2007). Type I IFNs include IFN α/β secreted by the leucocytes in the Atlantic salmon and induces antiviral defences in neighbouring cells, protecting them from becoming infected (Berg, et al., 2009). IFN α/β are transcriptionally activated by viral infection (Robertsen, 2006), (figure 3.). The secretion of IFNs acts quickly after viral entry as a “first line of defence” immune response that stimulates or informs the recipient cells through the IFN receptor (IFNR). The trans membrane IFNR precedes a signalling cascade via the Jak-STAT pathway, illustrated in figure 3.



modified from robertsen et al., 2006.

Figure 3. A simplified model of the type I Interferon system (IFN-system), illustrating the viral induction of IFN after viral recognition of the cell pattern recognition receptors (PRR). Recognition of ISAV in the endoplasm activates IRF3 by phosphorylation. IRF3 mediates to the nucleus together with Nf-kB and binds the IFN promoter that activates IFN production. The secreted IFN informs neighbouring cells in the form of a sos-signal that activates antiviral protein providing an antiviral state in the cell, in addition to further IFN production.

Jak kinases activate STAT1 and STAT2 through phosphorylation (Garcia-Sastre & Biron, 2006). The activation of STAT1 and STAT2 subsequently trigger a large number of IFN-stimulated genes (ISGs) (Robertsen, 2006). Which results in the production of antiviral proteins that protects surrounding cells from viral infection. There are several well studied

antiviral proteins for mammals (Katze, et al., 2002), while the myxovirus resistance proteins (Mx) and IFN-induced stimulated gene (ISG) 15 are known antiviral protein identified in the Atlantic salmon genome (Robertson, 2011).

The IFN- γ from type II IFN is also shown in Atlantic salmon (Kileng, et al., 2009) that increases the ability of the major histocompatibility complex (MHC) class I to present the antigen to T cells in the acquired immune response (Robertson, 2006). Moreover, IFN- γ secreted by macrophages or dendritic cells activates the acquired immune system in the maturation of dendritic cells and activation of natural killer cells (Stetson & Medzhitov, 2006). This indicates that IFNs has a crucial role in regulating innate immune responses, and also have factors in the acquired immunity.

1.3.1 The acquired immune response in Atlantic salmon upon viral infection and vaccination

As the name suggests the adaptive immune system is not active at birth but has to be stimulated by antigens to develop. Former antigen structures or virus fragments from vaccines are memorized by the acquired immune system to facilitate the elimination of the antigen upon a re-infection. The acquired immune system in Atlantic salmon is thought to be well developed and as the MHC class I and II was localized (Grimholt et al., 2003). Upon viral infection the acquired immune response includes lymphocytes, B cells and T cells in addition to a cascade of other cells. Antigen stimulated B cells becomes mature B cells. These mature B-cells have metabolized an antigen and present fragments of the antigen at the cell surface. Furthermore, B-cells produce memory cells involving the cell-mediated immunity or become a plasma cell, both essential elements of the humoral immunity. Intracellular pathogens as virus are often regulated by the cell-mediated immunity rely on the MHC I molecule. The MHC I molecule sits on the surface of all nucleated cells and present antigens to a matching T-cell and their T-cell receptors (TCR). MHC I involves the cytosolic pathway that comprehends the intracellular immune response against virus. The cytosolic pathway activate T helper (TH₁) involved in the cellular response stimulating production of memory cells (William, 2008).

1.4 Recognition of viral RNA and signalling pathways

The ISAV enters the host cell through receptor-mediated endocytosis. This is followed by the fusion of the virus membrane and the endosome membrane, resulting in release of ISAV in the endosome compartment. PRRs distinguishes viral ssRNA from other nucleic acids or nucleic acid fragments that are not normally found in the endosome (Haller, et al., 2006). Toll-like receptors are PRRs that recognize viral single and double stranded RNA as well as DNA. The Toll-like receptors; TLR 3, TLR 7 and TLR 8 are all important for viral recognition in the endosome compartments, see table 1. TLRs have a trans membrane part through the endosome membrane and a cytoplasmic part in the cytoplasm, see figure 4.

Table 1. Recognition of foreign nucleic acids by PRRs

Location	PRRs	Viral nucleic acids
Endosome	TLR3	dsRNA
Endosome	TLR7/8	ssRNA
Endosome	TLR9	DNA

As illustrated in Figure 4, Toll-like receptors activate the IFN system through specific signalling pathways to induce the gene expression of antiviral proteins.

The IFN system regulation in mammals is initiated by TLR 7/8 and TLR 9 both inducing the Interferon type 1 response by a signalling pathway through MyD88 (figure 4.), referred to as the “MyD88 dependent pathway” (Hacker, et al., 2006). Another point of view is that MyD88 primary induces the interleukin expression (Leichtle, et al., 2009). The TLR3 on the other hand strictly signals through the TriF adaptor molecule to induce type I interferon (IFN) expression and has recently been found to recognize ssRNA from Influenza A virus (Le Goffic, et al., 2006). Furthermore, IRF7 is thought to help IRF3 in the IFN production (Honda & Shimada, 2005). Moreover, the induction of IFN triggers transcription of IRF7A that enhance the production of IFN in a positive feedback loop, suggesting that even small viral stimuli are sufficient for a strong activation of the IFN-signalling (Haller, et al., 2006). The IRF3 and IRF7 are even thought to be activated in the same way (Iwamura, et al., 2001).

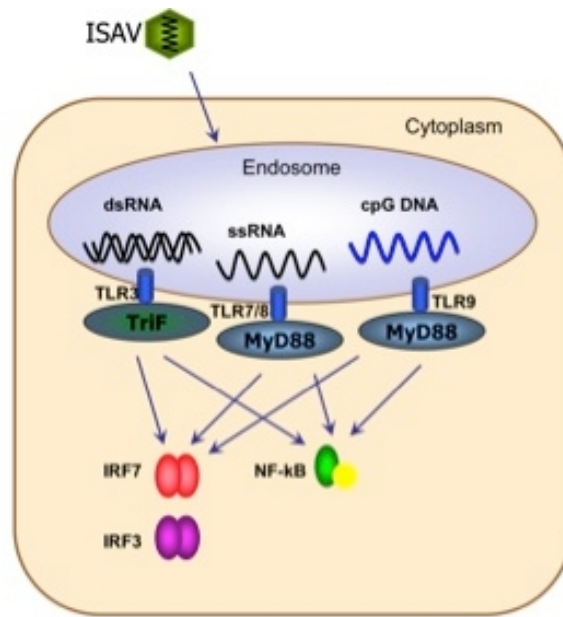


Figure 4. The recognition of viral nucleic acids by TLRs, and the TLR signalling pathways through adaptor molecules. TLR3 specifically signal through TriF while TLR7/8 and TLR9 signal through MyD88 as adaptor molecules for further signalling of antiviral response.

1.5 Other Orthomyxoviruses with IFN-antagonistic genes

The IFN system of the host cells that produce and secrete IFN upon the recognition of viral nucleic acids, play a crucial role in innate immune responses against virus infections in vertebrates (Haller, et al., 2007). To counteract the host defence, the ISAV segment 7ORF1 & 8ORF2 like other orthomyxoviruses have developed IFN antagonistic activity, an unknown mechanism that allows the virus to escape the hosts immune response (Garçla-Rosado et al., 2008). The equivalent IFN suppressor of the Influenza viruses' is the non-structural 1 (NS1) protein that also counters the establishment of the IFN antiviral state. The mechanisms behind the NS1 suppression of IFN are not clear but the RNA-binding of NS1 is though to have an essential role inhibiting IRF3 (Talon, et al., 2000). The ML-protein of the Thogoviruses is also hypothesized as an IFN antagonistic protein (Hagmaier, et al., 2003) and demonstrated to inhibit virus-induced activation of IRF3, but different from that of NS1 (Jennings, et al., 2005). Furthermore, characteristics of ML are distinct from the NS1, because ML is a structural protein.

1.6 Coiled-coil interactions

A motif found regarding IFN antagonistic activity was a Coiled-coil located in the C-terminal of the ISAV 8ORF2 segment (García-Rosado et al., 2008). No structural studies have shown the folding of the ISAV 8ORF2, but coiled-coils are known to form in dimeric structures (Qin, et al., 2003). Other protein interactions that were investigated in this master project were the previously characterized IRF3s of the Atlantic salmon (Bergan, et al., 2010). For mammals, the C-terminal dimerization of IRF3 is responsible translocation in to the cell core and binding of the IFN promoter, see figure 5. Yet, less is known about the interactions where the IRF3 of the Atlantic salmon is involved.

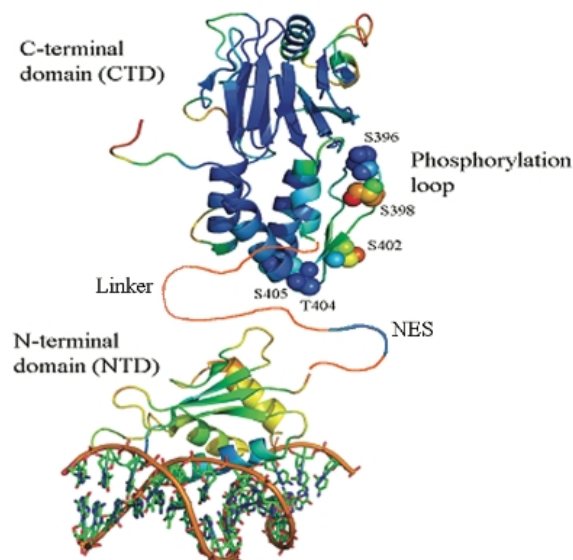


Figure 5. Illustration of the structure of IRF3 that is activated by phosphorylation. In the C-terminal a dimer structure is formed, assured to be essential for the N-terminal DNA binding domain to bind the IFN promoter (Dragan, 2007).

The IRF3 involved in the IFN system is activated upon virus infection by phosphorylations in the C-terminal transactivation domain (CTD). Phosphorylated IRF3 translocate to the nucleus where the coiled coil motifs of CTD provides dimerization and enables IRF3 to bind the IFN promoter as a dimer (Dragan, et al., 2007). The totally negative charge of the non-activated IRF3 seems to be an important difference between this and the activated IRF3, as illustrated in figure 6.

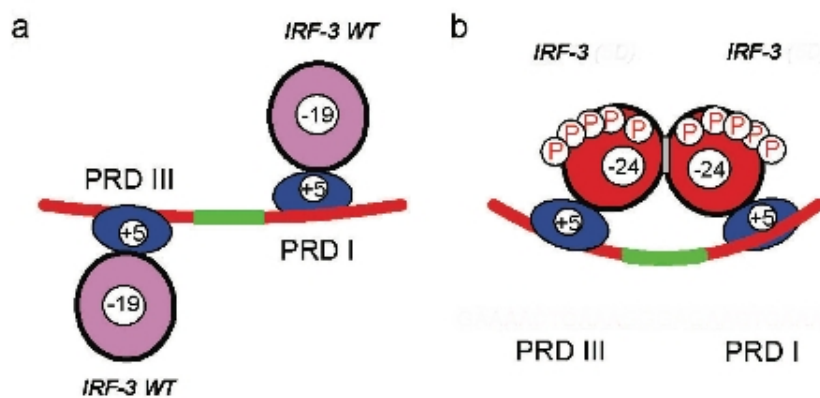


Figure 6a. Illustration of the binding of monomeric wild type (WT) IRF3 to the PRDI and PRDIII sites of the IFN promoter. **Figure 6b.** Binding of the phosphorylated, i.e. activated dimeric IRF3 to the PRDI and PRDIII sites of the IFN-B promoter (Dragan, et al., 2007).

1.7 Aim of study

A long-term goal of this project is to provide detailed understanding of the mechanisms that enables ISAV to replicate in the Atlantic salmon cell. To do this the ISAV interactions with the host's antiviral immune defence was studied. This is important in order to develop new methods for preventing and controlling ISAV that causes an emerging threat to the Atlantic salmon farming industry, as the present preventations like ISAV vaccines are not efficient enough.

In this thesis the main objectives was to explore protein-protein interactions committed between the ISAV and the interferon system of Atlantic salmon. This was achieved using Yeast-two-hybrid (Y2H) screening for interactions and co-immunoprecipitation (co-IP) for verification of the Y2H findings.

The work included the following tasks:

- Investigate protein-protein interactions between the ISAV proteins
- Study protein interactions between key proteins of the Atlantic salmon interferon type I system and the ISAV proteins responsible for IFN antagonistic activity.

Chapter 2. Key Molecular Techniques

In this project we have investigated interactions between ISAV proteins and salmon immune-related proteins. First, a screening was done using the Yeast-two-Hybrid technology, and second potential strong interactions were further explored by co-immunoprecipitation studies. This chapter gives an overview of these two key molecular techniques.

2.1 Yeast-two-Hybrid (Y2H)

The yeast-two-hybrid (Y2H) technology was developed by Fields & Song, 1989, in the late 1980s as a method for detection of protein–protein interactions. The Y2H uses the GAL4 transcriptional activator of the yeast *Saccharomyces cerevisiae*. Today the method is routinely used for large-scale identification of protein-protein interactions. The detection of interactions in Y2H is based on the activation of downstream reporter genes. As illustrated in Figure 7, these are activated when the DNA binding domain (BD) and the activation domain (AD) of the transcription factor, fused to the proteins of interest, are brought together. The BD is the domain responsible for binding to an upstream activation sequence (UAS) and the AD is the domain responsible for the activation of transcription (Fields & Song, 1989). The UAS binding triggers the transcription of a reporter gene, which makes yeast colonies grow on selective agar. Y2H vectors are designed to express “bait” genes in the BD vector, and the other gene of interest, the “prey”, in the AD vector. The “bait” is often a known protein while the “prey” is usually an unknown protein. These vector constructs are transformed and expressed in two separate *Saccharomyces cerevisiae* yeast strains that lack the biosynthesis of certain amino acids. When grown on media that lacks these nutrients, the yeast fail to survive.. See figure 7 for an illustration of the Y2H principle.

The Y2H technology has developed and is now used both for the screening of expression libraries for protein-protein interactions and also to study interactions between candidate proteins (Estojak, et al., 1995). This last approach is stated to provide less interference of false positives.

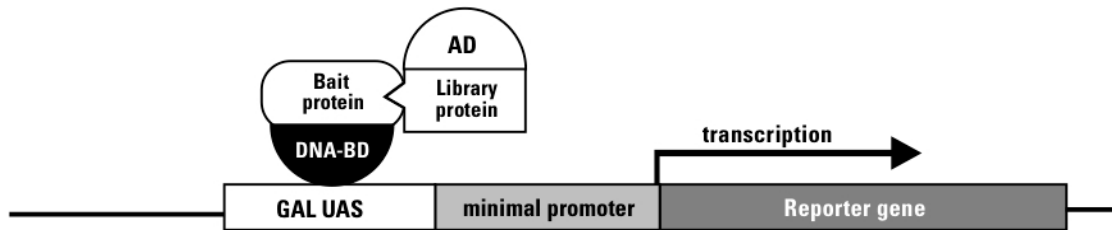


Figure 7. The yeast-two-hybrid principle is initiated by an interaction between the activation domain (AD) “prey” protein and the binding domain (BD) “bait” protein. This fulfils the characteristics of the BD as a transcriptional factor. The BD protein binds to the upstream activating sequence UAS for downstream transcription of the reporter gene.

Selective media are used to positively select for the cells that host a successful interaction between the bait and prey proteins and to test the stringency of the interaction. This involves culturing the transformed yeast on media lacking amino acid nutrients. Selective media lacking two amino acids, Leucine and Tryptophan, are called double dropout (DD) media. Selective media lacking Leucine, Tryptophan, and Histidine is referred to as triple dropout medium (TDO), while the media lacking Leucine, Tryptophan, Histidine, and Adenine is known as quadruple dropout medium (QDO). Growth on DD media indicates that the mating of prey and bait has occurred, growth on TDO indicates a weak interaction, while growth on QDO represents a strong interaction between bait and prey (James, et al., 1996).

Findings generated by Y2H are known to contain both false negatives and false positives caused by an overexpression of fusion proteins in the yeast nucleus. False negatives are generated by non-specific interactions while false positives are generated by detection of protein-protein interactions in yeast cells that in reality are never present in the same cell (Deane, et al., 2002). This high rate of false findings suggests that interactions found using the Y2H technique should be verified with other assays, such as co-immunoprecipitation (Pedersen, et al., 2007).

2.2 Co-Immunoprecipitation (Co-IP)

Co-immunoprecipitation (co-IP) is widely used for analysis of protein-protein interactions. The method of co-IP includes several steps. First, the vectors expressing the proteins X and Y are transfected in a host cell so that a possible interaction between X and Y can occur. The detection of proteins by co-IP is performed after cell lysis, which preserve the interaction. Next, an antibody against a specific target protein is coated on a beaded support such as Dynabeads (Invitrogen Dynal As, Oslo, Norway) to make an antibody-bead complex. This complex is incubated with the sample containing the proteins X and Y. The antibody-bead complex will then bind to the protein antigen directly or to an expressed tag coupled to the antigen, arranging an antigen-antibody-bead complex (figure 8). This complex is precipitated and proteins not precipitated are discarded.

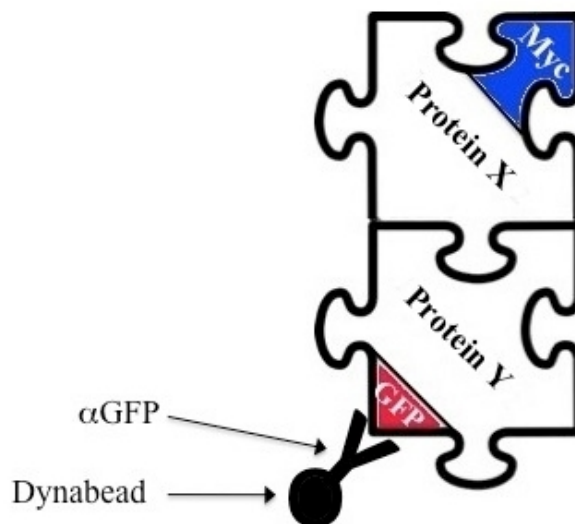


Figure 8: A simplified picture of co-precipitated proteins. Here the protein tags green fluorescence (GFP) is used for precipitation sometimes referred to as pull-down of the protein-protein interaction between protein X and protein Y.

The proteins and the antibodies are removed from the bead solid support (figure 9) using denaturing buffer and boiling. The proteins are identified through further analysis of the protein tags or antigens using SDS-PAGE in combination with Western Blot. The advantage of using tagged proteins is that you can use the same antibody for detection of several proteins while the protein antigen detection requires a unique antibody. However, tags might disturb the native function of the protein and other proteins might even bind the tag (Phizicky & Fields1995).

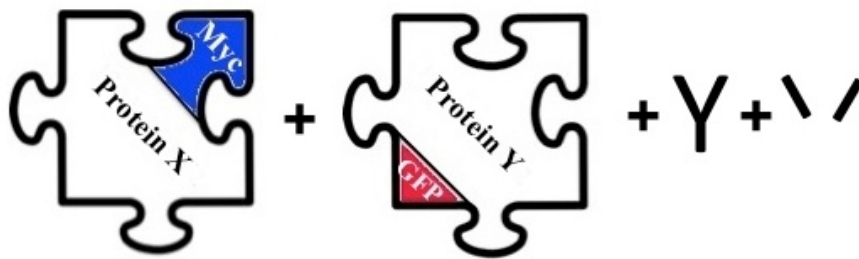


Figure 9: A simplified picture of lysed co-precipitated interaction complex contains fragments of α -antibodies and separates protein Y from protein X.

Immunoprecipitation can be performed with single precipitations (IP) focusing on the antigen, while co-IP focuses on the protein interacting with the antigen protein. Co-IP performs a "pull down" with the Dynabead-antibody-complex, which binds to a known protein in a protein complex. This "pull down" also enables the identification of other members of the protein complex. However, co-IP studies may require several rounds of precipitation with different antibodies because proteins in complexes could hide the antigen/tag. Due to this, some studies have focused on first screening for protein interactions with Y2H and then verification of the findings from Y2H using co-IP with two known candidate proteins. For validation of the protein-protein interactions the use of proper controls is essential to assure the exclusion of false positive findings (Sambrook & Russel 2001).

Chapter 3 Materials

3.1 Chemicals

Table 2: List of chemicals and their suppliers with catalogue number

Name	Supplier
DNA ladder 1kb	New England Biolabs, Ipswich, MA, USA. Cat. No. N3232L
DOBA (powder)	MP Bio medicals, Illkirch, France. Cat. No. 4026-032
Dulbecco's Modified Eagle's Medium (DMEM)	Lonza, Verviers, Belgium. Cat. No. BE12-614F
Ethanol	Arcus kjemi As, Vestby, Norway. Cat. No. 4196
Foetal Bovine Serum (FBS)	Invitrogen, Auckland, New Zealand. Cat. No. 10270-106
Gentamycin Sulphate (DMEM Ab)	Lonza, Verviers, Belgium. Cat. No. 17-518L
Immune-star WesternC kit	BioRad, 2000, Hercules CA, USA Cat. No. 170-5070
Isopropanol	Arcus kjemi As, Vestby, Norway. Cat. No. 300665
L-Glutamine 200mM in 0,85% NaCl	Lonza, Verviers, Belgium. Cat. No. BE17-605E
L-Histidine (His), ≥98%	Sigma, St. Louis, MO, USA. Cat. No. H-8125
Lipofectamine 2000	Invitrogen Carlsbad, CA, USA. Cat. No. 11668-027
Methanol 2,5L	Merck, Darmstadt, Germany. Cat. No 603-001-00-X
Milk powder	Tine, Oslo, Norway
OptiMEM	Invitrogen, Auckland, New Zealand. Cat. No. 31985-047
Ponceau S solution 0,1%	Sigma life science, St. Louis, MO, USA Cat. No. P7170
Precision Plus protein, dual colour standard, 500ul	BioRad, Hercules, CA, USA. Cat. No. 161-0374
Precision Plus protein, protein Western C standard, 250ul	BioRad, Hercules, CA, USA. Cat. No. 161-0376
Quadruple Dropout (QDO) media, lacking Leu, Trp, His and Ade	Clontech Laboratories, Mountain View, CA, USA Cat. No 630428
SOC medium	Invitrogen, Auckland, New Zealand. Cat. No. 15544-034
Trypsin EDTA buffer 200mg/L	Lonza Verviers, Belgium. Cat. No. BE17-161E
Yeast media lacking Leucine (Leu)	Clontech Laboratories, Mountain View, CA, USA. Cat. No. 630414
Yeast media lacking Tryptophan (Trp)	Clontech Laboratories, Mountain View, CA, USA. Cat. No. 630413
Triple Dropout (TDO) media, lacking Leu, Trp	MP Biomedical, Illkirch, France, Cat. No. 4530-

and His	122
XT Mops Criterion running buffer 20 x	BioRad, Hercules, CA, USA. Cat. No. 161-0788
Dynabeads Protein G	Invitrogen Dynal As, Oslo, Norway Cat. No. 100-04D
GelRed Nucleic Acid Gel Stain, 10,000X in water	BIOTIUM, Hayward, CA. Cat. No. 41003

3.2 Kits and enzymes

Table 3: List of Kits, enzymes, buffers and their suppliers with catalogue number

Name	Supplier
Frozen-EZ Yeast transformation II kit	Zymo Research, USA. Cat. No. T2001
Gateway BP Clonase II Enzyme Mix	Invitrogen, Carlsbad, USA. Cat. No. 11789-020
Gateway LR Clonase II Enzyme Mix	Invitrogen, Carlsbad, USA. Cat. No. 11791-020
Pfu Ultra II Fusion HS DNA Polymerase	Stratagene, La Jolla, CA, USA. Cat. No. 600670
Proteinase K solution	Invitrogen, Carlsbad, USA. Cat. No. 25530-049
Quick gel extraction and PCR purification Combo kit	Invitrogen, Carlsbad, USA. Cat. No. K220001
Western C solution kit	BioRad, Hercules, CA, USA. Cat. No. 170 5070
PureLink HiPure Plasmid DNA Midiprep Kit	Invitrogen, Carlsbad, USA. Cat. No. K2100-14
Zymoprep Yeast Plasmid Miniprep II	Zymo Research, Irvine, CA, USA. Cat. No. D2004

3.3 Vectors, primers

Table 4: List of vectors and their suppliers with catalogue number.

Name	Supplier
pENTER directional Topo cloning	Invitrogen, Carlsbad, USA. Cat. No. K2400-480
pDEST-Myc expressions vector, (UIT modified) from pcDNA-DEST47 vector	Invitrogen, Carlsbad, USA. Cat. No. 12281-010
pDEST-GFP expression vector (pDEST-47)	Invitrogen, Carlsbad, USA. Cat. No. 12281-010
pGBKT7-BD Vector, (pGBD) Binding domain, (fusion binding) c-Myc tag Kanamycin resistance	Clontech, Laboratories, Mountain View, CA, USA. Cat. No. 630443
pGADT7 AD Vector, (pGAD) Activation Domain, (fusion activation domain) HA tag Ampicillin resistance	Clontech, Laboratories, Mountain View, CA, USA. Cat. No. 630442
pDONR207 vector	Invitrogen, Carlsbad, USA. Cat. No. 12536017

Table 5: List of sequences primers. Invitrogen delivered all primers.

Application	Primer Name	Primer Sequence
Y2H expression vector	pGAD_fwd	GTCAAAGACAGTTGACTGTA
	pGAD_rev	GAGTCACTTTAAAATTTGTAT
	pGBD_fwd	TATTCGATGATGAAGATACC
	pGBD_rev	AAAACCTAAGAGTCACTTTAA
IP expression vector	pDEST-GFP_fwd	TGGAGTTCGTGACCGCCG

3.4 Solutions

Table 6: Solutions with recipes

Solutions	Recipe
Blot buffer (tris, glycine) 20% MeOH	30g. tris base, 144g Glycine and 800ml distilled H ₂ O
Glycerol for yeast construct -80C stocks	Heart Infusion Broth 2,125g, Distilled H ₂ O 85ml and Glycerine 15ml were mixed and aliquot in 2ml tubes.
Milliwater	Reverse osmosis and UV treated H ₂ O
Nonidet-P40 (NP40) lysis buffer	13.7ml 50mM NaCl, 1.0% NP-40 and 6.67ml 50mM Tris. Mixed by magnetic sterer, filled to 500ml with with distilled H ₂ O. pH adjusted til 8.0.
YEPD	10g Yeast extract, 20g Peptone and 20g Glucose were mixed and filled up with distilled H ₂ O till 1liter.
3x Sample buffer (SB) with 100mM DTT	7,2ml 1M Tris-Cl, 9ml 20% Sodium Dodecyl Sulfate (SDS), 9ml 100% Glycerol, and 0,018g Bromophenol blue, were mixed with distilled H ₂ O to a volume of 30ml. The pH was adjusted to 8,3.
LB-agar Ampicillin 100 µg/ml	37g LB-agar were dissolved and adjusted with distilled H ₂ O til 1 litre. The solution were mixed with magnetic stearer, autoclaved.and cooled to 50°C before adding 100mg Ampicillin. The liquid was poured into 10cm plates and the plates were stored at 4°C when set.
LB-agar Kanamycin 50 µg/ml	The same as above but adding 50mg Kanamycin for resistance.
LB-liquid media	5g yeast extract, 10g tryptone, 10g NaCl and 15g bacto agar were dissolved and adjusted with distilled H ₂ O till 1 litre. The solution were mixed with magnetic stearer, autoclaved and cooled to 50°C before stored at 4°C
Quadruple dropout (QDO) DOBA agar	43,7g DOBA, 0,59g Leu/Trp/His/Ade were dissolved and adjusted with distilled H ₂ O till 1 litre. The solution were mixed with magnetic stearer,

	autoclaved and cooled to 50°C poured into 10cm plates and the plates were stored at 4°C when set
Triple dropout (TDO) DOBA agar	The same preparation as above but adding 0,62g Leu/Tarp/His, instead of the above aminoacids.
Double dropout (DD) DOBA agar	The same preparation as above but adding 0,62g Leu/Trp/His + 0,2g Histidine.
Double dropout liquid (DD) DOBA media	Same as for DD agar but 27g DOB were used instead of DOBA
Single dropout Leucine	27g DOB 0,69g Leu were dissolved and adjusted with distilled H ₂ O till 1 litre. The solution was mixed with magnetic steerer and autoclaved.
Single dropout Tryptophan	Same as above but 0,74g Trp were used.
Phosphate- buffered saline (PBS) de Boer	Na ₂ HPO ₄ x 2H ₂ O 1,34g, NaH ₂ PO ₄ x H ₂ O 0,34g and NaCl 8,5g and filled up with distilled H ₂ O till 1liter. The solution was mixed with magnetic steerer and autoclaved.
Phosphate- buffered saline (PBS) with 0,1% Tween 20	NaCl 8 g, KH ₂ PO ₄ 0,2 g, Na ₂ HPO ₄ x 2H ₂ O 1,49 g, KCl 0,2 g, and 1ml tween 20 were mixed and filled up with distilled H ₂ O till 1liter. The pH was adjusted till 7,5.
10 X TBE buffer	55g Boric acid, 40ml EDTA and 107g Tris base were mixed and filled up with distilled H ₂ O till 1liter. The pH was adjusted till 8.

3.5 Biological material

Virus

Table 7: Virus, all are ISAV4 isolates (Glesvær outbreak), provided by NVI. (Markussen et al., 2008)

Segment	Protein	GenBank access number (complete cds)
1	PB2	DQ785178
2	PB1	DQ785192
3	NP	DQ785206
4	PA	DQ785220
5	F	DQ785234
6	HE	DQ785248
7	Non-structural protein 1 and 2	DQ785262
8	Non-structural protein and matrix protein genes	DQ785276.1

Innate immune system sequences from Atlantic salmon

Table 8: List of characterized genes of Atlantic salmon innate immune system sequences with accession number to GenBank, (the Gateway plasmid constructs with table 8 gene sequences incorporated, where also provided by UIT).

Gene (<i>Salmo salar</i>)	GenBank accession number	Reference
Interferon regulatory factor 3 (Irf3)	FJ517643 (IRF3)	(Bergan, et al., 2010)
Interferon regulatory factor 7 (Irf7a)	EU153263 (IRF7A)	(Kileng et al., 2009)
Interferon regulatory factor 7B (Irf7b)	FJ517644 (IRF7B)	(Bergan, et al., 2010)
LOC100302030 TLR8-like (Trl8)	NM_001161693	(Skjæveland, et al., 2009)
Myeloid differentiation primary response gene (88) (myd88)	EF672332	(Skjæveland et al., 2009)
Stat1 <i>Salmo salar</i> stat1a mRNA, complete cds (Stat1a)	GQ325309	(Skjesol, et al., 2009)
Tlr9 toll-like receptor 9 (Trl9)	NM_001123653	(Skjæveland, et al., 2008)
TIR-domain-containing adapter-inducing interferon- β (TriF)		Unpublished

Cell lines

Table 9: List of Cell lines and their suppliers with catalogue number

Cell line	Supplier
Human Embryo Kidney (HEK) 293T Cells	ATTC, NY, USA. Cat. No. CRL-11268
PJ69-2A <i>Saccharomyces cerevisiae</i>	Clontech, Laboratories, Mountain View, CA, USA.
Subcloning Efficiency DH5 α cells	Invitrogen, Carlsbad, USA. Cat. No.18265-017
Y187 <i>Saccharomyces cerevisiae</i>	Clontech, Laboratories, Mountain View, CA, USA.

Antibodies

Table 10: List of Antibodies and their suppliers with catalogue number

Antibody	Supplier
Goat anti Mouse –HRP 1:4000	Invitrogen. Eugene, Oregon, USA. Cat. No. 62-6520
Goat anti Rabbit –HRP 1:2000	Invitrogen. Eugene, Oregon, USA. Cat. No. G21234
Mouse anti Hemagglutinin (HA) 1:250,	Invitrogen. Eugene, Oregon, USA. Cat. No. 326700
Mouse anti-c-Myc Antibody, 1:5000	Invitrogen. Eugene, Oregon, USA. Cat. No. 13-2500
Precision Protein StrepTactin-HRP Conjugate,	BioRad, Hercules, CA, USA. Cat. Cat. No. 161-0380
Rabbit anti Hemagglutinin (HA) 1:500	Invitrogen. Eugene, Oregon, USA. Purified, Cat. No. 71-5500
Rabbit anti-GFP Antibody 1:2000	Invitrogen, Eugene, Oregon, USA. Cat. No. A-11122

3.6 Laboratory equipment

Table 11: List of technique, instrument and their suppliers

Technique	Instrument	Producer
Centrifugation	CS-6R Microfuge 22R	Beckman coulter, Pasadena, CA, USA. Beckman coulter, Pasadena, CA, USA.
Conventional PCR	DNA Engine peltier thermal cycler	MJ research, Waltham, MA, USA
Immunoprecipitation	Cell culture plates, 6well	Corning incorporated. Corning, NY, USA. Cat. No. 3506
Incubation	Shaker S3 Unimax 1010DT integrated with Unimax 1000 4.1.11 Steri-cycler 37°C CO2-incubator Digital dry bath D1200	Medkjemi AS, Norway. Heidolph, Schwabach, Germany. Thermo, Mariette, Ohio, USA. Labnet, Woodbridge, NJ, USA.
Microscopy	Fluorescence microscope LEICA DM IL Stereomicroscope	LEICA microsystems, Wetzlar, Germany. Leitz Labovet, Wetzlar, Germany
RNA/DNA analysis	Nano drop 2000 spectrophotometer	Thermo
Western blot	Immobilon Molecular Imager ChemiDoc XRS+ Western Blot chamber Blotting chamber Powerpac 200 Criterion XT precast SDS-PAGE gel, 10% Bis-Tris, 18well	Millipore Corporation Bedford, MA, USA, cat no IPVH00010 BioRad, Hercules, CA, USA. BioRad, Hercules, CA, USA. BioRad, Hercules, CA, USA. BioRad, Hercules, CA, USA. BioRad, Hercules, CA, USA Cat. No. 345-0112

3.7 Software

Table 12: List of software and sources

Name	Source
BLAST	(Altschul, et al., 1990) http://blast.ncbi.nlm.nih.gov/
CLC Main Workbench	(Weaver, et al., 2009) Aarhus, Denmark.
Expasy	(Gasteiger, et al., 2003) http://ca.expasy.org/tools/pi_tool.html
Multicoil	(Wolf, et al., 1997) http://www.osc.edu/supercomputing/software/apps/multicoil.shtml
Paircoil	(Berger, et al., 1995) http://groups.csail.mit.edu/cb/paircoil/paircoil.html

Chapter 4. Methods

4.1 Gateway cloning of Atlantic salmon and ISAV genes

Gateway cloning was used for preparation of expression vectors later used to screen for protein-protein interactions with Yeast-two-hybrid, followed by verification of the findings using co-immunoprecipitation. Both methods require candidate genes cloned in suitable Gateway vectors, these procedures were maintained in an attempt to find key interactions responsible for the Interferon antagonistic characteristics of ISAV (García-Rosado et al., 2008).

4.1.1 Expression vectors

This study focuses on proteins that have been hypothesized to suppress the innate immune system in mammals. Nearly all of the expression vectors that express these proteins and enable *in vivo* protein-protein interaction analysis in eukaryotic cells were provided by NVI and UIT. The ISAV 4 (*Glesvær outbreak*) isolate segment inserts are listed in table 7, section 3.5. They were provided by the NVI in suitable vectors for both Yeast-two-hybrid (Y2H) and Immunoprecipitation (IP). Vectors with inserts of expressed salmon immune-related proteins (table 8, section 3.5) were provided by the UIT, in suitable vectors for both Y2H and IP. In order to complement the provided ISAV inserts that were not expressed, ISAV segments 5 and 6 were made Gateway cloning compatible for this study. Initially, primers were designed for cloning into the pENTR vector.

4.1.2 Primer design for Gateway cloning

The initial step towards getting each ISAV segment into the Gateway compatible entry vector was to design primers for directional cloning into the pENTER/D-TOPO vector (Invitrogen, Invitrogen, Carlsbad, USA.). The pENTR vector was used as an entry clone to Gateway

cloning enabling vector inserts in suitable vectors for protein interaction analysis, see figure 10. The directional cloning ensures the proper orientation of the inserts by adding 5' CACC upstream of the ATG initiation codon in the forward primer that base pair with the overhang sequence GTGG in the pENTR vector. A stop codon was incorporated in the end of the reverse primer if the inserted gene did not have one. To compliment the not expressed vector insert from the ISAV membrane segments 5 and 6, primers were designed for these segments without the trans-membrane region. The primers are shown in table 13.

Table 13. Oligonucleotide primers used for PCR amplification and cloning of the ISAV membrane genes

Primer name	Nucleotide sequence (5'-3')
HE_FWD	<u>CACCATGG</u> CACGATTCATAATTTTATTC
HE_REV	TTAATTC CC CAGATGTTGCTGC
HE_REV	TTAAGCTGAACCATGTAGTGAG
F_FWD	<u>CACCATGG</u> CCTTTTCTAACAATTTTAGT
F_REV	TTAACCTCCACCCTAATCCCTT
F_REV	TTAACCGCCTCAAGGGCTCCTT

Sequence representing recognition sites at the segment 5(F) and 6(HE). The underlined sequence is necessary for directional cloning that base pair with GTGG in the pENTR vector. The bold letters represent start and stop codons.

4.1.3 Polymerase chain reaction (PCR) for the pENTR vector

The next step towards cloning ISAV segments in Gateway compatible vectors was to amplify the ISAV inserts 5(F) and 6(HE) from a DNA plasmid, listed in table 7, section 3.5. The amplification was performed by PCR using the PfuUltra II fusion HS kit, (Stratagene La Jolla, CA, USA.). The PCR reaction volumes and the PCR program are showed below in table 14.

Table 14: PfuUltra II fusion HS reaction volumes

Distilled water (dH ₂ O)	40.5 µl
10× PfuUltra® II reaction buffer	5.0 µl
dNTP mix (25 mM each dNTP)	0.5 µl
DNA template (100 ng/µl)	1.0 µl
Primer #1 (10 µM)	1.0 µl
Primer #2 (10 µM)	1.0 µl
PfuUltra® II fusion HS DNA polymerase	1.0 µl
Total reaction volume	50.0 µl

PCR reaction was run on the DNA Engine peltier thermal cycler (MJ Research, Waltham, MA, USA) using the PCR program outlined beneath in table 15.

Table 15: Conditions for PfuUltra II fusion HS amplification

<i>Number of cycles</i>		<i>Temperature</i>	<i>Duration</i>
1	Activation	95°C	2 minutes
30	Denaturation	95°C	20 seconds
	Annealing	55°C	20 seconds
	Elongation	72°C	15 seconds
1		8°C	∞

The PCR reaction products were run on an agarose gel electrophoresis separating the amplicons depending on nucleotide size. The procedure is described in the following text.

Agarose gel electrophoresis

1. A 1,5% agarose gel with 1xTBE buffer was prepared and the agarose dissolved by heating in microwave.
 2. The Gel red, (BIOTIUM, Hayward, CA. USA.) was added to the solution for a 1:10 000 dilution.
 3. The solution was poured into a gel mould with a comb, after the gel had stiffened the comb was removed and the gel was placed in a gel chamber.
 4. The samples were mixed in a loading buffer (6:1) and loaded into the gel chambers made by the comb, together with 1kb DNA ladder.
 5. The gel was run at 90Volt for 60minutes.
 6. Visualizing of the PCR fragments on the gel was performed using the UV light application of ChemiDoc XRS+ (BioRad, Hercules, CA, USA.).
-

If multiple bands were amplified the fragment of expected size was cut out from the gel and purified with a gel extraction kit (Quick gel extraction, Invitrogen, Carlsbad, USA.). The procedure is described stepwise below.

Gel extraction, (Invitrogen, Carlsbad, USA).

1. Gel pieces of the wanted PCR fragment with a weight > 400mg were incubated with the Gel solubilisation buffer (L3) in a water bath at 50 °C for at least 15minutes, dissolving the gel piece. The gel solution was loaded into the centre of a PureLink® Clean-up Spin Column and placed in a 2ml tube.
2. A Centrifugation step at $>10,000 \times g$ for 1 minute was followed by discarding the flow-through and adding 50 μ l wash buffer (W1). This was followed by another centrifugation step at $>10,000 \times g$ for 1 minute. The flow-through was discarded.
3. To remove any residual wash buffer and ethanol, centrifugation at maximum speed was performed for 2–3 minutes. The wash tube was discarded and the PureLink Spin Column placed into an elution Tube.
4. For elution 50 μ l elution buffer (E1) was added to the centre of the PureLink Spin Column placed in a new tube and incubated for 1 minute at room temperature. A centrifugation step at $>10,000 \times g$ for 1 minute, obtained the purified DNA in the new tube. The DNA was stored at -20°C

When a single concentrated PCR product of the desired length was visualized after gel electrophoresis, PCR purification was used (PCR purification Combo kit, Invitrogen, Carlsbad, USA.). The procedure is described below.

PCR purification, (Invitrogen, Carlsbad, USA).

1. Four volumes of Binding Buffer (B2) were added to one volume of PCR reaction and mixed well.
 2. The PureLink Clean-up Spin Column was placed in a 2ml tube. The solution was centrifuged in room temperature at $10,000 \times g$ for 1 minute, the flow through discarded.
 3. Then, 650 μ L of Wash Buffer was added to the PureLink Spin Column. The solution was centrifuged in room temperature at $10,000 \times g$ for 1 minute, then the flow-through was discarded.
-

4. At last, an additional centrifugation step at maximum speed, 2–3 minutes at room temperature, was performed to remove any residuals.
5. Samples were then ready for elution by the elution Buffer (50 μ L), which was added to the centre of the PureLink® Spin Column, placed in a new tube. The samples were incubated at room temperature for 1 minute. This was followed by centrifugation at maximum speed for 1 minute, to obtain the purified PCR product. The purified DNA was stored at 20°C.

The isolated and purified PCR products were inserted into the pENTR vector as an entry clone for Gateway cloning. That enables desired inserts in suitable vectors for protein-protein interaction analysis with Y2H and IP analysis.

4.1.4 Cloning into pENTR D-TOPO vector (Entry clone)

The pENTR cloning enables further site-specific recombination cloning. It takes advantage of the site-specific recombination properties of bacteriophage lambda (Landy, 1989), that recombinants the entry clone insert (gene) of interest into suitable vectors, as illustrated in Figure 10.

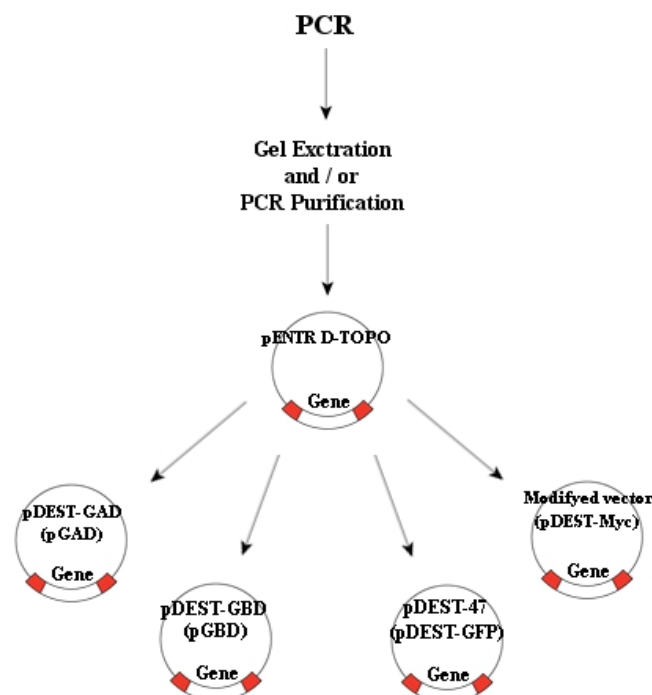


Figure 10. Gateway cloning starting with the pENTR as an entry clone that enables the cloning of gene inserts to a destination (pDEST) vector. The four different kinds of pDEST vectors were used as expression vectors for protein-protein interactions studies.

These vectors were used for transfection into Y2H cells (Y187/ PJ69) for Y2H analysis, and into human cells (HEK293T) for co-IP analysis. All plasmid constructs were sequenced (Macrogen, Korea), and verified with CLC Main Workbench.

Expression vectors pGBD and pGAD were transfected into *Saccharomyces cerevisiae* cell line PJ69-2A and Y187, both from Clontech Laboratories (Mountain View, CA, USA). The pGAD and pGBD transfections were used for Yeast-two-hybrid (Y2H) analysis. For the IP analysis, the expression vectors pDEST-GFP and pDEST-Myc were transfected into Human embryonic kidney 293T cells (ATTC, NY, USA). The transfer of gene inserts between pENTR vector and pDEST vector was performed using recombination by LR Clonase™ II enzyme (Invitrogen, Carlsbad, USA.). The LR reaction procedure is listed below.

The LR Reaction

1. In a 1,5ml tube 1 µl of the pENTR and 1 µl of the pDEST vector along with 8 µl TE buffer were added at room temperature, followed by easily mixing the solution by pipetting.
2. The LR Clonase™ II enzyme was put on ice along with the samples and the vector solution, for about 2 minutes.
3. The LR Clonase II enzyme was mixed briefly using vortex, before 2 µl of LR Clonase II enzyme mix was added to each reaction and mixed by vortex briefly.
4. Briefly centrifugation to gather the liquid in the bottom of the tube was followed by incubation of the reactions at 25°C for 1 hour.
5. To terminate the reaction 1 µl of the Proteinase K solution was added, briefly vortexed and incubated at 37°C for 10 minutes.

All pDEST vectors with a gene of interest inserted were designed as expression vectors and transformed in to DH5α Subcloning efficiency cells (Invitrogen, Carlsbad, USA) for the expression of the desired genes. The procedure is described below.

Transformation of Gateway vectors

1. The DH5 α were thawed on wet ice together with 1,5ml tubes for pre cooling, 1 μ l of DNA was added to 25 μ l DH5 α cells and gently mixed. Cells and DNA were incubated on ice for 2minutes.
2. The cell solution was heat shocked in 30s at 42°C and quickly incubated on ice for 2 minutes.
3. The next step was adding 250 μ l SOC medium to the solution, which was then incubated on 225rpm shake at 37°C for 1hour.
4. Finally, 50 μ l of the transformation mix was plated on LB plates containing 100 μ g/ml kanamycin for the pENTR D-TOPO, and the pGBD vector. LB plates with 100 μ g/ml ampicillin were used for the pDEST vectors and the pGAD vector. The LB plates were incubated at 37°C overnight before the bacterial colonies with the wanted gene inserts incorporated were selected for further investigation.

Preparation of pDEST vectors

The plasmid preparation of pDEST vectors was performed with the PureLink HiPure Plasmid DNA Midiprep Kit (Invitrogen) resulting in high quality plasmid DNA. Starting from 25 mL overnight E. coli colonies cultures transformants were picked from an appropriate resistance agar plates.

1. Bacterial culture cells were harvested by centrifugation at 4,000 \times g for 10 minutes, the residual medium was discarded.
 2. Resuspension Buffer (R3) with RNase A was added to an amount of 10 mL to the cell pellet for resuspension of the cell pellet.
 3. For cell lysis, 10 mL Lysis Buffer (L7) was added and mixed gently by inverting the capped tube until the lysate mixture was homogenous. The lysate was incubated at room temperature for 5 minutes.
 4. 10 mL Precipitation Buffer (N3) was added, and mixed by inverting the tube until the solution was homogeneous.
-

-
5. The precipitated lysate was transferred into the HiPure Filter Midi Column and then passed through the filter by gravity flow. The flow-through was discarded.
 6. After the HiPure Filter Midi Column stopped dripping, the filtration cartridge was removed and discarded.
 7. The midi column was washed with 20 mL wash buffer (W8) and the flow-through was discarded.
 8. For the elution of DNA a new tube was placed under the HiPure Filter Midi column, and 5 mL elution buffer (E4) added. The flow-through contains the DNA.
 9. Precipitation of DNA was performed by adding 3.5 mL of Isopropanol (Arcus, Hummelstown, PA, USA) to the DNA. The DNA-isopropanol mixture was incubated for 2 minutes at room temperature.
 10. The DNA-isopropanol mixture was centrifuged at $12,000 \times g$ for 30 minutes at 4°C . and the supernatant was discarded.
 11. The DNA pellet was resuspended in 3 mL 70% ethanol (Arcus, Hummelstown, PA, USA), and centrifuged at $12,000 \times g$ for 5 minutes at 4°C . The supernatant was carefully removed and discarded.
 12. The pellet was air-dried for approximately 10 minutes.
 13. The DNA pellet was resuspended in 200 μL TE Buffer and the purified DNA stored at -20°C .

4.2. Screening for protein-protein interactions using Yeast two Hybrid (Y2H)

After the preparations of suitable expression vectors for Y2H was performed the actual study began with screening of protein-protein interactions. Screening were performed to identify novel protein-protein interactions between ISAV proteins and proteins from the innate immune system of Atlantic salmon. In addition protein-protein interactions within the ISAV proteins were analysed.

4.2.1 Expression vectors for Yeast-two-hybrid

Genes of interest regarding Interferon antagonistic activity were cloned into the pENTER D-TOPO vector (Kanamycin resistance), and subcloned by LR clonase recombination

(Invitrogen) into eukaryotic expression vectors. Modified Clontech vectors (pGADT7 and pGBKT7) were used (Pedersen, et al., 2007) and named the pGAD and the pGBD vector, shown in Figure 10. The pGAD vector was then suitable for Y2H analysis. The other Y2H vector, the pGBD, was constructed with the LR reaction from pENTR to pDONR207 vector with Gentamycin resistance for selection. The BP reaction was used to recombine the insert back to pENTR, followed by LR reaction into pGBD. The BP reaction procedure is listed below.

The BP Reaction

1. In a 1,5ml tube 1 μ l of the pENTR and 1 μ l of the pDONR 207 vector along with 8 μ l TE buffer were added at room temperature, followed by easily mixing the solution by pipetting.
2. The BP Clonase TM II enzyme was put on ice along with the samples and the vector solution, for about 2 minutes.
3. The BP Clonase II enzyme was mixed briefly with vortex, before 2 μ l of BP Clonase II enzyme mix was added to each reaction and mixed by vortex briefly.
4. Incubation of the reactions at 25°C were made for 1 hour after a briefly centrifugation to gather the liquid in the bottom of the tube.
5. To terminate the reaction 1 μ l of the Proteinase K solution were added, briefly vortex and incubated at 37°C for 10 minutes.

The pDONR 207 pathways are needed because both pENTR and pGBD have kanamycin resistance that makes it hard to distinguish the pENTER colonies from pGBD colonies on the agar plate after transformation. Now, the pGBD vector and the pGAD vector were picked as colonies after transformation from Kanamycin and Ampicillin resistance LB plates, respectively. The pGBD expression vectors were then prepared by minipreparation as the other pDEST vectors in section 4.1.4. Further applications towards Y2H analysis the Y2H expression vectors were transfected into yeast cells, the procedure is listed below.

Transfection of Yeast PJ69-2A and Y187 *Saccharomyces cerevisiae* strains

1. Preparation of competent *Saccharomyces cerevisiae*, PJ69-2A and Y187 were performed by growth of the cells at 30°C in 10ml YEPD broth to mid log phase (OD600 of 0.8-1.0)

-
2. The overnight cells-culture was pelleted at 500x g for 4 minutes and supernatant discarded, followed by washing the cells with 10ml of EZ 1 solution.
 3. The cells were re-pelleted and supernatant discarded, followed by resuspending the pellet in 1ml of EZ2 solution.
 4. Competent yeast cells were mixed with 0,2-1 µg DNA, PJ69-2A for the pGBD vector and Y187 for the pGAD vector, and 500µl EZ 3 solution and incubated at 30 °C for 45minutes in a 225rpm shake incubator.
 5. The transfection mixture was spread in an amount of 150 µl on DOBA plates, lacking leucine for pGAD, and lacking Tryptophan for pGBD, and incubated for 2-4 days.

For storage, sequencing and the Yeast-two-hybrid study, preparation of yeast plasmids were performed by Zymoprep™ Yeast Plasmid Miniprep II (Zymo Research), as described below. The enzyme Zymolase used for yeast plasmid preparation has a specific lytic digestion of *Saccharomyces cerevisiae* cells.

Yeast Plasmid preparation

1. Aliquots of 1.0 ml of the full-grown yeast cells was added into 1.5 ml tubes and centrifuged at 600x g for 2 minutes, the supernatant was discarded.
 2. Solution 1 in an amount of 150 µl was added to each pellet, in addition 2 µl of Zymolyase was added, and the pellet was resuspended by easily vortex.
 3. The samples were incubated at 37°C for 60 minutes.
 4. The Solution 2 was added in an amount of 150 µl and mixed well, followed by supplement of 150 µl Solution 3. Again the tubes were mixed well,
 5. Centrifugation of the solutions at maximum speed for 2 minutes.
 6. The supernatant where transferred to the Zymo-spin-1 column, the Zymo-spin-1 column was centrifuged at 600x g for 30 seconds and the flow-through was discarded.
 7. The Wash buffer was added to the column and in an amount of 550 µl and centrifuged at 600x g for 2minutes. The flow-through was discarded.
 8. For elution the Zymo-spin-1 column was put into a new 1,5ml tube and 10 µl of TE buffer was added to the column and centrifuged at 600x g for 30 seconds. The yeast plasmids were stored at -20°C.
-

4.2.2 Sequencing of the expression vectors for Yeast-Two-Hybrid

Primers pGADfwd, pGADrev and pGBDfwd, pGBDrev, were sent together with 30 μ l Y2H expression vectors in a concentration of 100ng/ μ l, to Macrogen, Korea, for sequencing. The primers are listed with their nucleotide sequence in Table 5. Section 3.3.

Table 15: Yeast-two-Hybrid expression vectors sent for sequencing

Vector	ISAV4 segment
pGAD	1
pGAD	3
pGAD	4
pGAD	7ORF1
pGAD	7ORF2
pGAD	8ORF1
pGAD	8ORF2
pGBK	2
pGBK	8ORF1
pGBK	IRF3
pGBK	STAT1
pGBK	TLR9
pGBK	IRF7

4.2.3 Protein expression analysis of Y2H-expression vectors by Western Blotting

For protein expression analysis, yeast cell lines PJ69-2A and Y187 transfected with pGBD vector and pGAD vector, respectively, were grown at 30°C in 10ml media lacking Tryptophan for PJ69-2A (pGBD) and media lacking Leucine for Y187 (pGAD) for 48hours. The cultured cells were centrifuged at 500g and the supernatant discarded. Then, the pellet was resuspended in 20 μ l 3X sample buffer (SB) with dithiothreitol (DDT) for yeast cell lysis. The samples were boiled for 15 minutes, quickly centrifuged, kept on ice and subjected to SDS-PAGE gel (Criterion XT precast SDS-PAGE gel, 10% Bis-Tris, 18well, BioRad, Hercules, CA, USA). The SDS-Page electrophoresis procedure is described below.

SDS-Page

1. The SDS-Page gel Criterion XT precast 10% Bis-Tris 18well (BioRad, Hercules, CA, USA), was prepared with 1x XT mops Criterion running buffer (BioRad, Hercules, CA, USA) in a SDS- Page electrophoresis chamber.
2. The samples were loaded together with the precision plus protein ladders, Dual colour standard, (BioRad) and the protein Western C standard, (BioRad).
3. All SDS-Page in this study, both for Y2H, IP and Co-IP, were run at 100V for 15minutes and an additional 150V for 1 hour.

The next step towards visualising the molecular size of the proteins encoded by the yeast expression vectors in yeast cells was to transfer the separated proteins on the SDS-PAGE, on to a blot membrane. The blot membrane was then hybridized with primary and secondary antibodies diluted in PBS with 0,5% Tween with 1% blocking agent for detection of the incorporated expressed tags. The procedure for blotting is outlined below.

Blotting

1. First, the blot membrane Immobilon Molecular Imager (Millipore Corp, USA), was prepared by exposure to Methanol (Merck, Darmstadt, Germany) for 5 seconds and incubated for 10 minutes in blotting buffer.
 2. The SDS-PAGE was immediately added to the blot membrane and packed with three blot papers and one fiber pad on each side, all pre-moisture with blot buffer and clamped in the blot sandwich. It was also ensured that no air bubbles had been formed between the gel and the membrane.
 3. The blot sandwich was applied between solid supports in the blot chamber filled with pre-cooled blot buffer. All blots in this study, both for screening and verification of protein-protein interactions, were run at 100V for 1 hour. During this procedure, the negative charged proteins migrates towards the positive electrode, but are stopped and bound by the blot membrane.
 4. To visualize the successfull transfer between the gel and the blot membrane, the blot membrane was placed in 0,1 % Ponceau S solution (Sigma life science, MO, USA), and washed in filtered water.
-

5. Unspecific background binding of antibodies was prevented using 5% non-fat milk (Tine, Oslo, Norway) diluted in PBS with 0,5% Tween 20, incubated during agitation at room temperature by Shaker S3 (Medkjemi AS, Norway), for 1hour at 200rpm.
6. The incorporated hemagglutinin (HA) tag and the Myc N-terminal tag for pGAD and pGBD respectively, were hybridized with primary Rabbit anti-HA antibody (Invitrogen, Eugene, Oregon, USA) diluted till 1:500, and Mouse anti-c-Myc antibody (Invitrogen, Eugene, Oregon, USA), both incubated at room temperature for 1 hour.
7. The blot membrane was washed in PBS 0.1% Tween 20 for 2x 15minutes at 200rpm by shake incubation in room temperature.
8. Secondary antibodies Goat anti Mouse –HRP 1:4000 (Invitrogen, Eugene, Oregon, USA) and Goat anti Rabbit –HRP 1:2000 (Invitrogen, Eugene, Oregon, USA), was applied for pGAD and pGBD respectively and incubated at room temperature for 1hour at 200rpm by shake incubation. The incubation mixture of the secondary antibody layer included 1:10000 dilution of the precision Protein StrepTactin-HRP Conjugate (BioRad, Hercules, CA, USA), for visualisation of the precision plus protein ladders.
9. The blot membrane was washed in PBS 0.1% Tween 20 for 2x 15minutes at 200rpm by shake incubation in room temperature.
10. The Western C solution (BioRad, Hercules, CA, USA) containing hydrogen peroxidase were used as a substrate to the conjugated HRP on the secondary antibody, the substrate catalyse the oxidation of luminol that emits luminescence. The chemiluminescence was detected in a Chemi Doc XRS+ (BioRad, Hercules, CA, USA).

4.2.4 Mating of the pGBD vector in PJ69-2A cells and pGAD vector in Y187 cells.

The plasmid constructs of pGBD vector in PJ69-2A and the pGAD vector in Y187, both two-hybrid (GAL4) compatible, were grown at 30°C for 48h. They were grown in selective media lacking Tryptophan for PJ69-2A with pGBD vector, and lacking Leucine for Y187 with pGAD vector. These cells were mated in pairwise combinations of PJ69-2A with the pGBD vector including genes of the Atlantic salmon immune system, and Y187 with pGAD vectors including clones of the viral segments of ISAV. The haploid transformants were mated using 500µl of each yeast cell strain and were then incubated at 30°C for 24 hours in rich YEPD media, providing a transcriptional assay for detecting protein interactions in vivo in yeast cells. The diploid transformants were plated in an amount of 150 µl on DOBA plates with double dropout (DD) media lacking Leucine and tryptophan (Clonetech Laboratories, CA,

USA), showing if mating has occurred. Growth of triple dropout medium (TDO) lacking Leucine, Tryptophan, and Histidine (Clontech Laboratories, CA, USA), indicates a weak interaction and quadruple dropout medium (QDO) lacking Leucine, Tryptophan, Histidine, and Adenine (Clontech Laboratories, CA, USA), indicating a stronger interaction.

4.2.5 Negative and positive control for Y2H

Plasmid constructs (pGAD/pGBD) lacking viral segment and salmon immune inserts were used as negative controls, while segment 8ORF2 together with 8ORF2 in pGAD and pGBD constructs were used as a positive control. J. Ramsell, Ph.D at NVI, established these controls.

4.3. Co- Immunoprecipitation (Co-IP)

Co-immunoprecipitation studies were performed for further investigation of the novel protein-protein interactions found during Y2H analysis. The expression vectors pDEST-Myc (Invitrogen, Carlsbad, USA) with ISAV segment inserts was provided by the NVI, while the expression vectors pDEST-GFP, (Invitrogen, Carlsbad, USA) for the IRFs were provided by the UIT.

4.3.1 Expression vectors for co-Immune precipitation.

Primer design, amplification, purification cloning- and transformation procedures related to expression constructs used for co-IP studies are described in section 4.1. The vectors utilized for detection of protein-protein interactions are the pDEST-Myc vector with N-terminal Myc tag and the pDEST-GFP with C-terminal GFP tag.

4.3.2 Sequencing of the expression vectors for co-immunoprecipitation

The sequence primer pDEST-GFP_fwd shown with nucleotide sequence in section 3.3 were sent to Macrogen, Korea for sequencing, along with the expression vectors in a concentration of 100ng/μl, listed in Table 16.

Table 16: Immunoprecipitation expression vectors sent for sequencing

Vector	Interferon regulatory factor
pDEST-GFP	3
pDEST-GFP	7a
pDEST-GFP	7b

4.3.3 Positive and negative controls for Co-IP

The co-IP of the pDEST-Myc with ISAV segment 8ORF2 insert (pDEST-Myc 8ORF2) and the pDEST-GFP with ISAV segment 8ORF2 insert (pDEST-GFP 8ORF2), were used as positive controls as J. Ramsell, Ph.D at NVI, has established. The segment 8ORF2 is known to homodimerize (Manuscript in preparation). The negative IP controls of the pDEST-Myc 8ORF2 from single transfectants, as well as the Co-IP of the (pDEST GFP STAT1 – pDEST Myc 8ORF2) was used. The last control mentioned were shown negative in Y2H screening analysis, see table 19.

4.3.4 Transfection of HEK 293T cells for IP

Three days before the IP, the HEK 293T cells (ATTC, NY, USA) were plated so that the cells would be 90% confluent and at log phase the day of transfection. First, cells were washed in 4ml phosphate buffered saline (PBS) and harvested in 2ml Trypsin EDTA buffer 200mg/L, (Lonza, Verviers, Belgium) until all cells were non-adherent. 10ml of DMEM with 2% L-Glutamine antibiotics (200mM) (Lonza, Verviers, Belgium) and 10% Foetal Bovine Serum, FBS, (Invitrogen, Auckland, New Zealand) were added and centrifuged at 1200g for 5minutes. The supernatants were discarded and the cell pellet re-suspended in DMEM without antibiotics and distributed in aliquots of 2,5ml in 6-well plates (Corning incorporated. Corning, NY, USA.).

Two days before the IP, the transfection of expression vectors pDEST-GFP and pDEST-Myc into 90% confluent HEK293 was performed, using Lipofectamine 2000, (Invitrogen).

The Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used for transfection of pDEST-myc expression vectors encoding viral protein with N-terminal c-Myc tag. The

pDEST-GFP expression vector encoding salmon IRF protein with C-terminal GFP tag were diluted to 4 μ g (2 μ g for Co-IP) and mixed with 250 μ l Opti-MEM (Invitrogen, Auckland, New Zealand.). 10 μ l lipofectamin 2000 diluted in 250 μ l Opti-MEM was incubated for 5 minutes and gently mixed with 250 μ l plasmid solution, before it was incubated at room temperature for 20 minutes. The transfection mixes (~500 μ l) were added to cells and incubated in a carbon dioxide incubator at 37°C for 48h. Transfected cells were inspected with a fluorescence microscope to look for GFP-fluorescent proteins.

Lysates of HEK293T cells co-transfected with Myc- and GFP-tagged constructs were prepared by first placing the cells on ice. The medium was removed and the cells were washed very gently twice in ice cold PBS. The cells were then flushed off with ice cold PBS and transferred to a precooled tube. Cells were centrifuged at 1200g for 2min, the PBS was removed and the cell pellet lysed in 250ul NP-40 lysis buffer with protease inhibitors. 20 μ l of the sample was saved for analysis of protein expression in the total cell lysate. The rest of the sample was used in co-IP. The cell lysate were kept at 20°C until the time of the analysis.

4.3.5 Transfection control

Fluorescence microscopy was used as transfection control to detect the expression of the pDEST-GFP vectors. Fluorescence microscopy is a non-destructive analysis method of biological molecules that uses fluorescence for specific detection of single molecules. Fluorescence is the detected photon emission from a molecule that absorb photons of a different wavelength, this phenomenon enables single molecule detection through fluorescence labelled biological material (Spring & Davidson, 2010). In this study the proteins encoded by pDEST-GFP expression vectors are labelled or tagged with the green fluorescence protein (GFP), and can be detected after transfection of HEK 293T-cells. GFP is susceptible for absorption of photons of 395nm and emission of green fluorescence at 507nm (Cramer, et al., 1996). Emission was detected in a LEICA DM IL Fluorescence microscope and photos of the transfection controls were taken using a Nikon DSMS digital sight camera.

4.3.6 Antibody binding to Dynabeads

For immunoprecipitation, the rabbit anti-GFP antibody (Invitrogen), in a dilution of 1:2000, was bound to Dynabeads (Invitrogen Dynal As, Oslo, Norway.) First, Dynabeads were resuspended by pipetting and 15µl beads per sample were transferred to a tube. The tube with beads was placed on a magnet and the supernatant discarded. 2µg of αGFP Antibody were dissolved in 200µl PBS with 0.01% Tween and added to the beads. The beads-Ab complex was incubated by rotation for 1h at 4°C. The tube was placed on magnet and the supernatant removed. The tube was removed from the magnet and the beads-Ab complex resuspended in 200µl PBS with 0.01% Tween.

4.3.7 Precipitation

The 230µl of total cell lysate was centrifuged for 10 min at 12 000 rpm at 4°C. The cleared lysate was transferred to a new tube, and kept on ice.

The tube with the beads-Ab complex was placed on the magnet and as much as possible of the supernatant was removed. The cleared lysate was added to the beads-Ab complex and incubated through rotation for 10minutes at RT to allow the antigen (Ag) to bind the Dynabead-Ab complex (1hour or more for low affinity Ab).

The tube was placed on a magnet and the supernatant was transferred to a new tube for further analysis if desired. The Dynabead-Ab-Ag complex was washed three times in 200ul of the non-denaturation NP40 lysis buffer and the supernatant re-suspended on magnet by gentle pipetting. Finally the dynabeads-Ab-AG complex was re-suspended in 100ul NP40 lysis buffer. To avoid co-elution of proteins bound to the tube wall, the bead suspension was separated on magnet into a new tube.

Each target protein was eluted from solid support by adding 40 µl of 3x SB + 100mM DTT, 3xSB with 100mM DTT was also added the total lysates. Both IP and total lysate samples were boiled for 5 min and quickly centrifuged to collect the sample at the bottom of the tube.

The samples were kept on ice before loading 20µl of them on the SDS-PAGE and letting the gel run at 100V for 15 minutes followed by 175V for 1 hour.

4.3.8 Negative and positive control for co-IP

Two negative controls were used in IP. The first is a single precipitation with 8ORF2 Myc. The second more strict negative control was STAT1, which was shown negative in Y2H, and used in co-IP as pDEST STAT1 GFP with the interacting target for positive control ISAV 8ORF2 Myc (pDEST STAT1 GFP - 8ORF2 Myc). The positive control was the pDEST 8ORF2 Myc - pDEST 8ORF2 GFP interaction found and established in co-IP by J. Ramsell, Ph.D at NVI.

4.3.9 Western Blotting for Co-IP

The method of western blotting for Co-IP was performed as for Western Blotting for Y2H, see section 4.2.3 for a detailed description. Exceptions from that procedure described in regard the procedure after antibody hybridisation, which is specific for the Co-IP. This is described below.

1. The incorporated c-terminal green fluorescence (GFP) and c-terminal Myc tag for pDEST-GFP and pDEST-Myc respectively, were detected with primary Rabbit anti-GFP Antibody diluted 1:2000 (Invitrogen. Eugene, Oregon, USA.), and Mouse anti-c-Myc Antibody, diluted 1:5000 (Invitrogen. Eugene, Oregon, USA.), for 1 hour.
2. The blot membrane was washed in PBS 0.1% Tween for 2x 15minutes at 200rpm by shake incubation. Secondary antibodies Goat anti Rabbit –HRP diluted 1:2000 (Invitrogen. Eugene, Oregon, USA.), and secondary Goat anti Mouse –HRP diluted 1:4000 (Invitrogen. Eugene, Oregon, USA.) were applied for pDEST-GFP and pDEST-Myc respectively. The incubation mixture of secondary antibody's included 1:10 000 dilution of Precision protein Strep Tactin-HRP conjugate (BioRad, Hercules, CA, USA), for visualisation of the precision plus protein ladders.
3. The blot membrane was washed in PBS 0,1 tween 20 for 2x 15minutes at 200rpm by shake incubation at room temperature.
4. The following steps with activation of the HRP enzyme and blotted protein visualisation by chemi luminescence are the same as described in section 4.2.3.

4.4 Bioinformatics

4.4.1 Comparison of Y2H and Co-IP vectors with genes from GenBank

Local BLAST and the nucleotide search algorithm BLASTn were used from (CLC Workbench, Aarhus, Denmark.). DNA query was searched for in the GenBank nucleotide database with default settings, the sequence of the vector inserts was translated to verify that the sequences were in frame (without stop codons).

4.4.2 Coiled-coil prediction.

The coiled-coil prediction was performed with coiled-coil prediction software programs available on internet; Paircoil (Berger, et al., 1995), and Multicoil (Wolf, et al., 1997).

4.4.3 Alignment of IRF3 from Human and Atlantic salmon

The human IRF3 is activated by phosphorylation first after viral infection. For prediction of a putative similar activation mechanism of the Atlantic salmon IRF3, a protein alignment was performed to look for evolutionary conservation of coiled-coil motifs found in human IRF3 (Qin, et al., 2003). This would suggest a similar regulation of the Atlantic salmon IRF3.

Chapter 5. Results

It has previously been shown that ISAV segments 7ORF1 and 8ORF2 proteins exert IFN antagonistic activity (García-Rosado, et al., 2008). To investigate where in the Atlantic salmon immune system the ISAV viral segments interact, we have screened for protein-protein interactions using the yeast-two-hybrid technology and further studied our findings with immunoprecipitation. The results of this study are here presented.

5.1 Screening for protein-protein interactions using Yeast-Two-Hybrid (Y2H)

Genes potentially involved in the mechanisms for IFN antagonistic activity during ISAV infection were chosen as candidates for a protein-protein interaction screening using the Y2H technology. Genes central in the interferon type I signalling cascade, such as TLR8, TLR9, IRF3, IRF7, MyD88, TRIF and STAT1 were inserted in pGBD vectors. The ISAV segments were inserted in pGAD vectors. These expression vectors were provided by Professor Jorunn Jørgensen, UIT. Dr. Jon Ramsell, NVI, cloned the ISAV genes into the pGAD-vectors.

5.1.1 Identifying the vector inserts used in Y2H experiments

All the inserts in expression vectors were sequenced to verify sequence identity – which is crucial for providing reliable results.

Y2H vector inserts were compared with genes from GenBank using a BLASTn search. The results showed that all expression vector inserts sequences matched the wanted nucleotides for gene expressions, shown in Table 17. This made them reliable for use in the Y2H protein-protein interaction screening analysis.

Table 17: Alignment of sequenced pGAD and pGBK vector nucleotides searched against GenBank

Query sequence	Hit description	E-value	Bit score	Query start	Query end	Identity	Gaps
pGAD_7ORF2_fwd	DQ785262 (ISAV 4, segment 7)	0	831	222	640	419	0
pGAD_7ORF1_fwd	DQ785262 (ISAV 4, segment 7)	0	1 560	164	950	787	0
pGAD_s4_fwd	DQ785220 (ISAV 4, segment 4)	0	1 564	162	950	789	0
pGAD_8ORF1_fwd	DQ785276 (ISAV 4, segment 8)	0	1 172	164	754	591	0
pGBK_8ORF1_rev	DQ785276 (ISAV 4, segment 8)	0	1 172	827	237	591	0
pGAD_S3_fwd	DQ785206 (ISAV 4, segment 3)	0	1 562	163	950	788	0
pGAD_8ORF2_fwd	DQ785276 (ISAV 4, segment 8)	0	1 441	158	888	730	0
pGBD_IRF7A_rev	EU153263 (<i>Salmo salar</i> IRF7A)	0	1 630	100	950	842	0
pGBD_STAT1_rev	GQ325309 (<i>Salmo salar</i> STAT1)	0	1 388	950	243	706	0
pGBD_TLR9_rev	NM_001123653 (<i>Salmo salar</i> TLR-9)	0	1 132	810	240	571	0
pGBK_IRF3_rev	FJ517643 (<i>Salmo salar</i> IRF3)	0	1 628	98	950	949	0

Expression vectors for use in Y2H were identified with E-values at zero, this indicates that the probability of finding a better match is zero. We controlled that these sequences were in a proper reading frame for translation using CLC Main Workbench.

The pGBD and pGAD-vectors were transformed into PJ69-2A and Y187 yeast cells respectively, using Frozen-EZ yeast transformation II (Zymo research). These PJ69-2A and Y187 cells were grown on selective media lacking Tryptophan and Leucine respectively. The molecular size of the vector inserts from the ISAV-expressed proteins were analysed on Western blot, illustrated in figure 11a and 11b.

5.1.2 Western blot for Y2H

The molecular size of the expression vectors used in Y2H were visualized by Western blotting, results are shown in figure 11a and 11b. This was enabled using the HA tag of pGAD, and the Myc tag of pGBD. Figure 11a shows the expressed pGBD-vectors, while figure 11b shows the expressed pGAD-vectors.

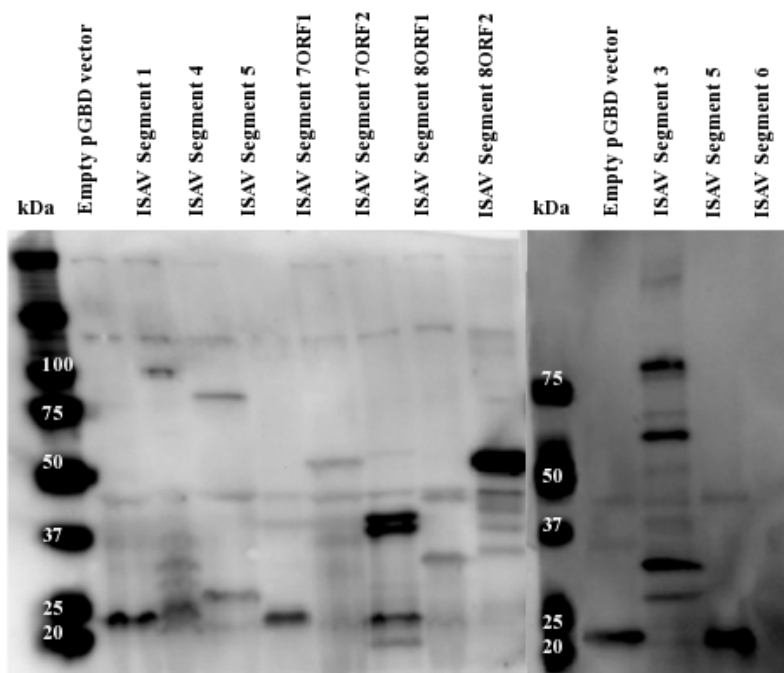


Figure 11a. SDS PAGE of expressed ISAV segments in pGBD vectors transformed into *Saccharomyces cerevisiae* PJ69-2A yeast cell strain (Clontech, Laboratories, Mountain View, CA, USA.).

ISAV segments inserts in pGBD with Myc-tag were blotted with rabbit-anti hemagglutinin (α HA) 1:500 (Invitrogen, Invitrogen. Eugene, Oregon, USA.), followed by goat-anti-rabbit – HRP 1:2000 (Invitrogen, Invitrogen. Eugene, Oregon, USA.).

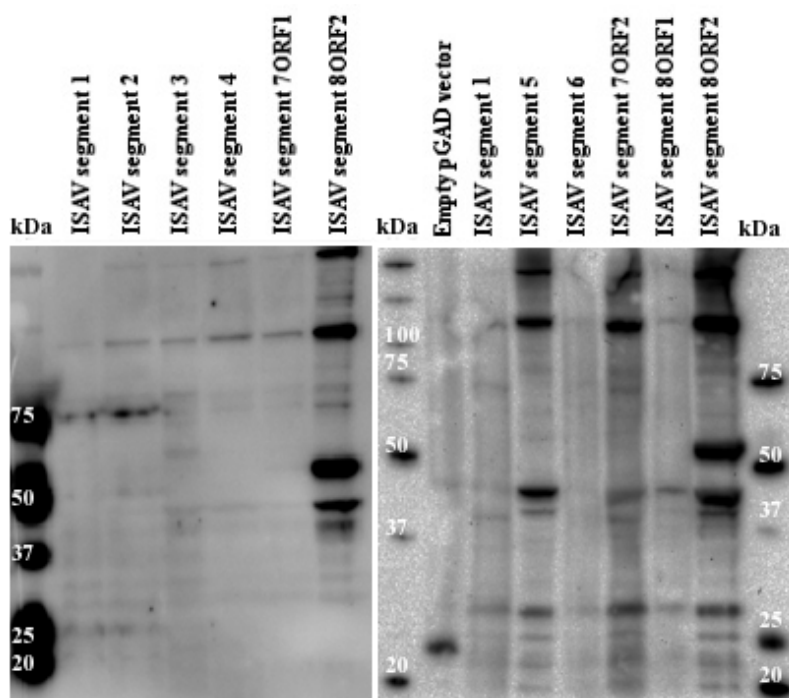


Figure 11b. SDS PAGE of expressed ISAV segments in pGAD vectors transformed into *Saccharomyces cerevisiae* Y187 yeast cell strain (Clontech, Laboratories, Mountain View, CA, USA.).

ISAV segment inserts in pGAD with a translating HA tag were blotted with mouse-anti-Myc 1:5000 (Invitrogen, Eugene, Oregon, USA.), followed by goat-anti-mouse–HRP 1:4000 (Invitrogen, Eugene, Oregon, USA.). The molecular size of the ISAV proteins compared to the predicted size (Appendix 1.), are shown in Table 18.

Table 18: Corresponding molecular size (kDa) of ISAV segment expressed proteins for Yeast-two-hybrid analysis

ISAV segment	1	2	3	4	5	6	7ORF1	7ORF2	8ORF1	8ORF2
pGBK (kDa)	78		67	66			35	17	22	25/37
pGAD (kDa)		77	67	65			35	17	22	25/37

The expressed ISAV proteins that corresponded to the predicted molecular size (Appendix 1) are marked in green. Non-corresponding ISAV-segment expressed proteins are marked in red.

The molecular size of “empty vectors” is 23kDa for both pGAD and pGBD, shown in figure 11a and 11b. The molecular size of these vectors lacking inserts is withdrawn from the Y2H vectors with an insert, and comparison to the predicted molecular weight (Appendix 1). The ISAV segment 2 pGBD was not expressed because the sequencing of the insert showed

insufficient results, for documentation see Appendix 2. To get all ISAV segments in both Y2H-vectors the initial steps with primer design, PCR and cloning of segment 5 and 6 into the pDEST-vector were performed, but due to lack of time the samples with non-corresponding molecular size were not studied further.

5.1.3 Yeast-Two-Hybrid mating

Proteins expressed from pGBD and pGAD-vectors that interact during mating enabled us to grow colonies on selective media. The stringency of the protein-protein interaction was measured in selective media, and to confirm that mating has occurred, double dropout medium (DD) was used. Triple dropout medium (TDO) indicates a protein-protein interaction, while quadruple dropout medium (QDO) indicates an even stronger protein-protein interaction. The first aim of this study was to identify possible protein-protein interactions between ISAV proteins. Only the interaction between 8ORF2 together with 8ORF2 was found. This interaction was used as positive control in further studies where the ISAV proteins were mated with proteins from the Atlantic salmon innate immunity. Results are shown in Table 19.

Table 19: Scoring of protein interactions between the innate immune system of the Atlantic salmon and the ISAV in the Y2H-model

pGBK/pGAD	Empty	Myd88	IRF3	IRF7a	STAT1	TLR8	TLR9	TriF	8ORF2
Empty	-	-	-	-	-	-	-	-	-
Seg.1	-	-	-	-	++	-	-	++	-
Seg.2	-	-	-	-	++	-	-	++	-
Seg.3	-	-	+++	-	-	-	-	++	-
Seg.4	-	-	-	-	++	-	++	++	-
Seg.5	-	-	-	-	-	-	-	-	-
Seg.6	-	-	-	-	-	-	-	-	-
7ORF1	-	++	++	++	++	-	-	-	-
7ORF2	-	-	-	-	-	-	-	-	-
8ORF1	-	-	-	-	-	-	-	-	-
8ORF2	-	-	+++	+++	-	++	-	-	+++

Growth was scored after 6 days, when the positive control 8ORF2 with 8ORF2 showed massive growth on quadruple dropout medium (QDO) lacking Leucine, Tryptophan, Histidine, and Adenine. +++ massive growth on QDO (a strong interaction); ++ massive growth on triple dropout medium (TDO) lacking Leucine,

Tryptophan, and Histidine (indicates a weaker interaction). Empty plasmid constructs with vectors lacking viral proteins or salmon immune clone were used as negative controls.

The protein-protein interactions between IRF3, IRF7A and ISAV segment 8 ORF2 are shown in figure 12.

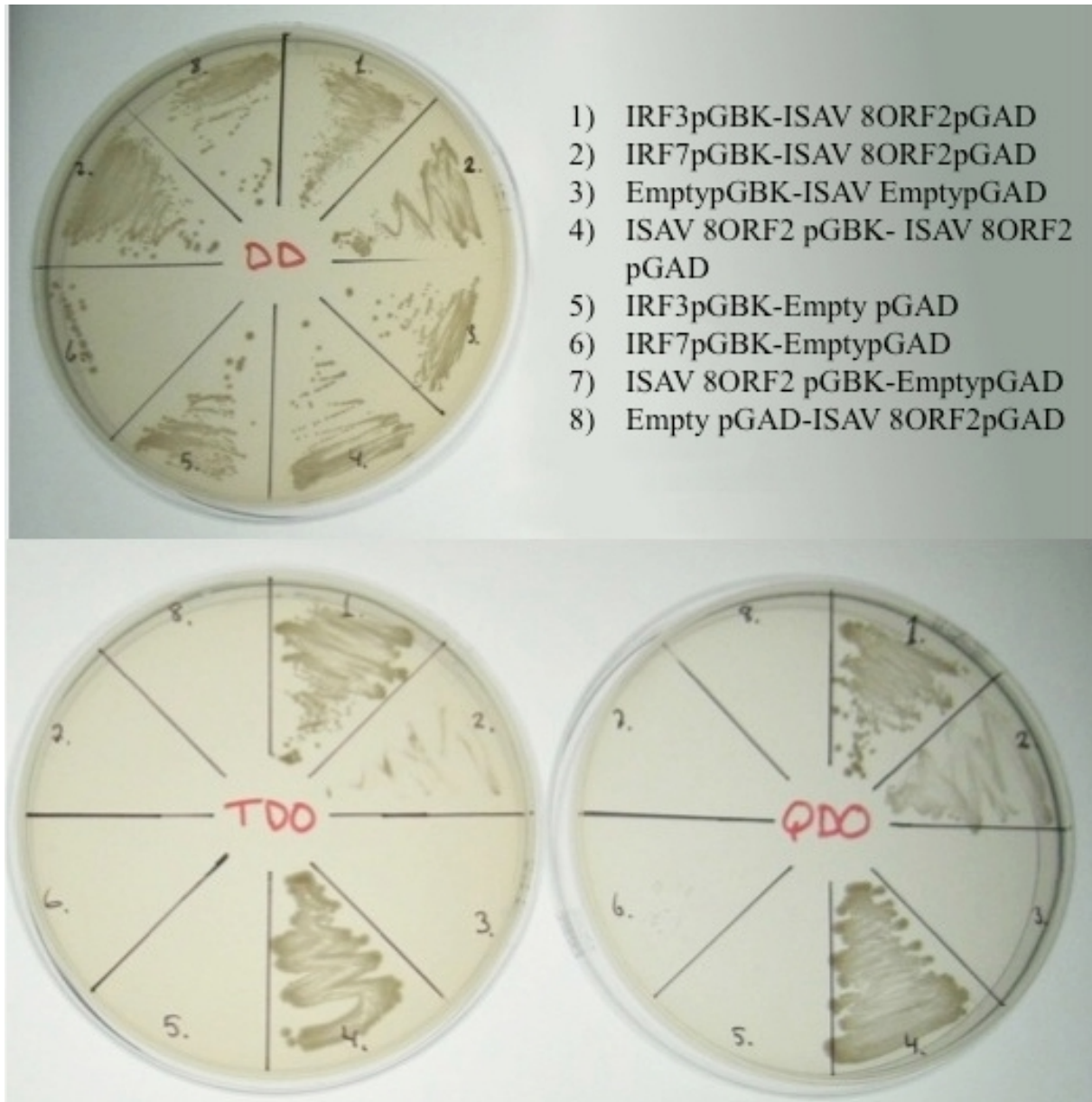


Figure 12. Growth of mated Y2H on selective media indicating the stringency of the protein-protein interactions. Plasmid constructs with vectors lacking viral proteins or salmon immune clone (3,5,6,7,8) were used as negative controls, while ISAV segment 8ORF2pGBD- ISAV segment 8ORF2pGAD (4) served as positive control.

The IRF3/IRF7a interaction with ISAV 8ORF2 was measured according to the stringency of growth on selective media; as these grew under all types of stringency, this indicates a strong

protein-protein interaction. The most interesting protein-protein interactions found during Y2H screening was investigated further using co-immunoprecipitation for verification of the findings.

5.2 Verification of protein-protein interactions using co-immunoprecipitation

The detected protein-protein interactions between (IRF3 - ISAV 8ORF2) and (IRF7a – ISAV 8ORF2), were chosen for further investigation using co-immunoprecipitation (Co-IP). Both are candidates to be involved the IFN antagonistic activity of ISAV. In this co-IP study, we used pDEST-Myc and pDEST-GFP as expression vectors. We also analysed an additional IRF7b insert in p-DEST-GFP, but decided to exclude it from further analysis. This because the analysis showed that the pDEST-IRF7a insert was equal to the IRF7a insert used in Y2H, which made it suitable for further study.

5.2.1 Identification of the pDEST –GFP vector sequence used in co-immunoprecipitation

The UIT provided the pDEST-GFP expression vector constructs with IRFs inserted. As illustrated in figure 13 and 14, these inserts were sequenced and matched against GenBank using BLASTn. The positive control vectors (pDEST 8ORF2-GFP - pDEST 8ORF2-Myc) were identified by Dr. J.Ramsell (data not shown).

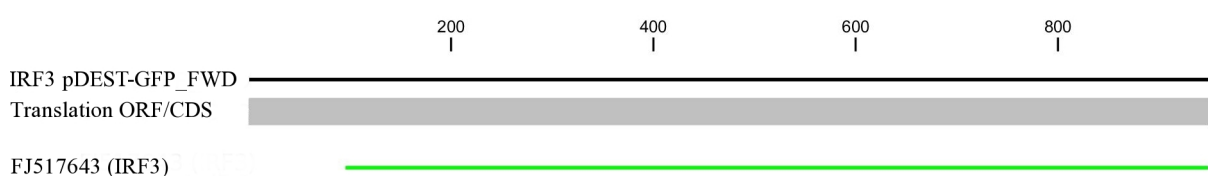


Figure 13: Local Blastn visualisation of IRF3 pDEST-GFP vector sequence as query and the best match of the local database with accession number FJ517643 (IRF3).

As shown in figure 13, IRF3 pDEST-GFP is identical (E-value=0) to the Atlantic Salmon IRF3 corresponding sequence from the GenBank. The result presented in figure 14 shows that IRF7a is equal to the Atlantic Salmon IRF7a sequence from the GenBank.

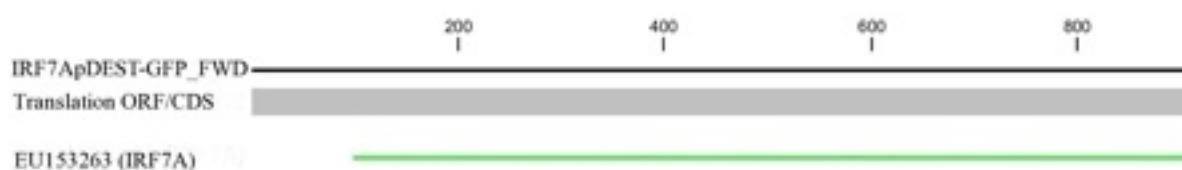


Figure 14: Local Blastn visualisation of the IRF7a pDEST-GFP vector sequence as query and the best match of the local database with accession number EU153263 (IRF7a).

The verification of the insert sequences of the expression vectors used in IP are summarised in Table 20. The results show that the insert sequences of the expression vector matched the wanted nucleotides for gene expressions.

Table 20: Alignment of sequenced pDEST-GFP vector sequences

Query sequence	Description	E-value	Bit score	- Query start	Query end	Identity	Gaps
IRF7A-GFP_FWD	EU153263(IRF7A)	0	1 629,99	100	950	842	0
IRF3-GFP_FWD	FJ517643.1(IRF3)	0	1 628,01	98	950	849	5

After validation of the vector inserts for the IP samples the pDEST-Myc and pDEST-GFP vectors were single-transfected or co-transfected into HEK 293T cells. The transfection step was also validated through transfection controls.

5.2.2 Transfection control by detection of fluorescence from translated pDEST-GFP vectors

For the transfection control we used the pDEST-GFP vectors expression of their c-terminal GFP tag, as visualized by fluorescence microscopy. The transfection efficiency is measured in the amount of emitted fluorescence. Figure 16 shows the transfection efficiency of the pDEST-GFP vector with IRF3 and IRF7a inserts as found in this study.

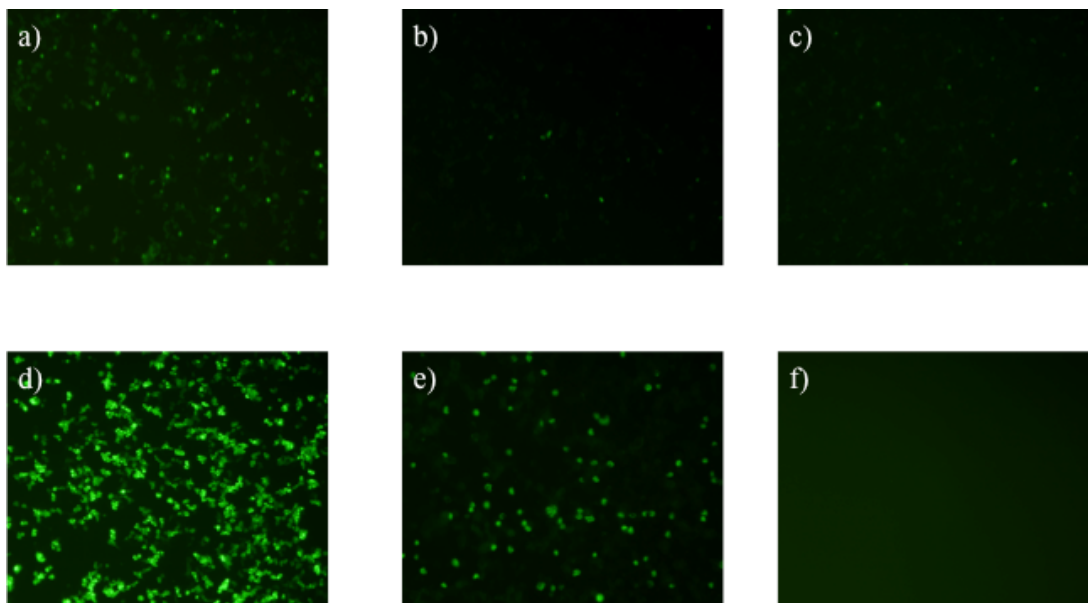


Figure 16. Transfection efficiency of single-transfected and co-transfected HEK293T cells visualised with fluorescence microscopy. a) ISAV 8ORF2 pDEST Myc - IRF3 pDEST GFP, b) ISAV 8ORF2 pDEST Myc - IRF7a pDEST GFP, c) ISAV 8ORF2 pDEST Myc - IRF7b pDEST GFP d) Positive transfection and immunoprecipitation control (ISAV 8ORF2 pDEST GFP - ISAV 8ORF2 pDEST Myc, e) Positive transfection and negative immunoprecipitation control STAT1 pDEST GFP - ISAV 8ORF2 pDEST-Myc, f) Negative transfection and precipitation control ISAV 8ORF2 pDEST Myc. a to e are co-transfections while f is a single-transfection.

5.2.3 Detection of Co-precipitated proteins on Western Blot

Western Blot was used for detection of co-IP with rabbit-anti-GFP antibody 1:2000 (Invitrogen, Eugene, Oregon, USA.). The ISAV proteins Myc tag and the IRF proteins GFP tag enabled the detection with α Myc antibody (Figure 17a) and α GFP antibody (Figure 17b), respectively.

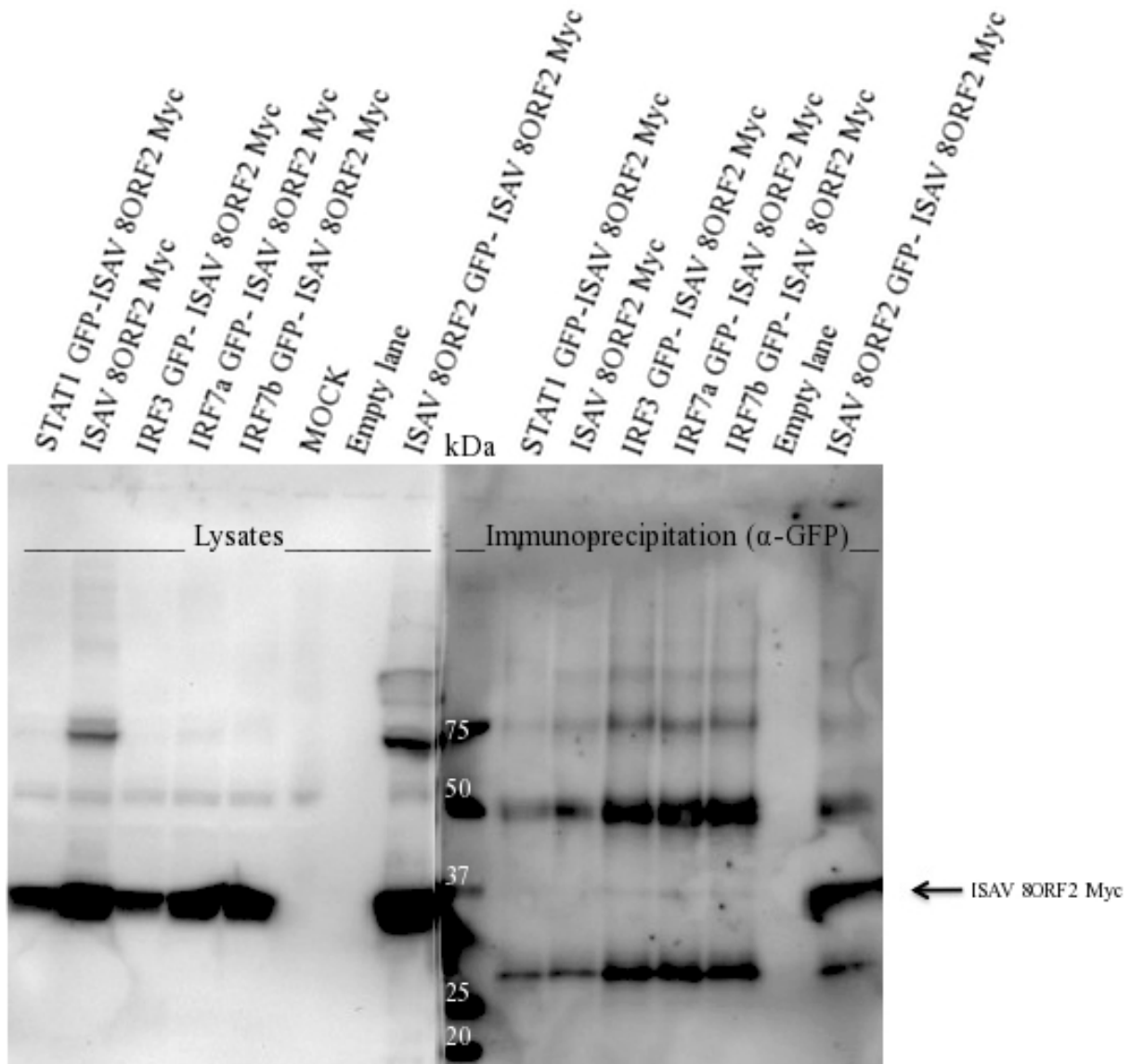


Figure 17a Detection of interactions between the analysed proteins using Western Blot of co-IP with α GFP precipitation incubated with α Myc antibodies, visualizing the Myc tag of precipitated proteins.

Immunoprecipitations were performed with α GFP pull-down and the incubation with primarily and secondary α Myc-antibodies was used for detection of Myc tagged proteins. The results for the positive control (ISAV 8ORF2 GFP – ISAV 8ORF2 Myc) showed a strong ISAV 8ORF2 Myc band at 37kDa. The estimated size of 27kDa for ISAV 8ORF2 matches well with its predicted molecular size (Appendix 1), as the size of the Myc tag itself is 10kDa. The results of the negative controls (STAT1 GFP- ISAV 8ORF2 Myc) and (ISAV 8ORF2 Myc) were both negative for ISAV 8ORF2 Myc. The results for the interactions found in Y2H, (IRF3 GFP – ISAV 8ORF2) and (IRF7a GFP – ISAV 8ORF2), showed weak bands for

ISAV 8ORF2 Myc. The antibody contamination bands at 50kDa and 30kDa showed the heavy and light chain of the precipitation antibody.

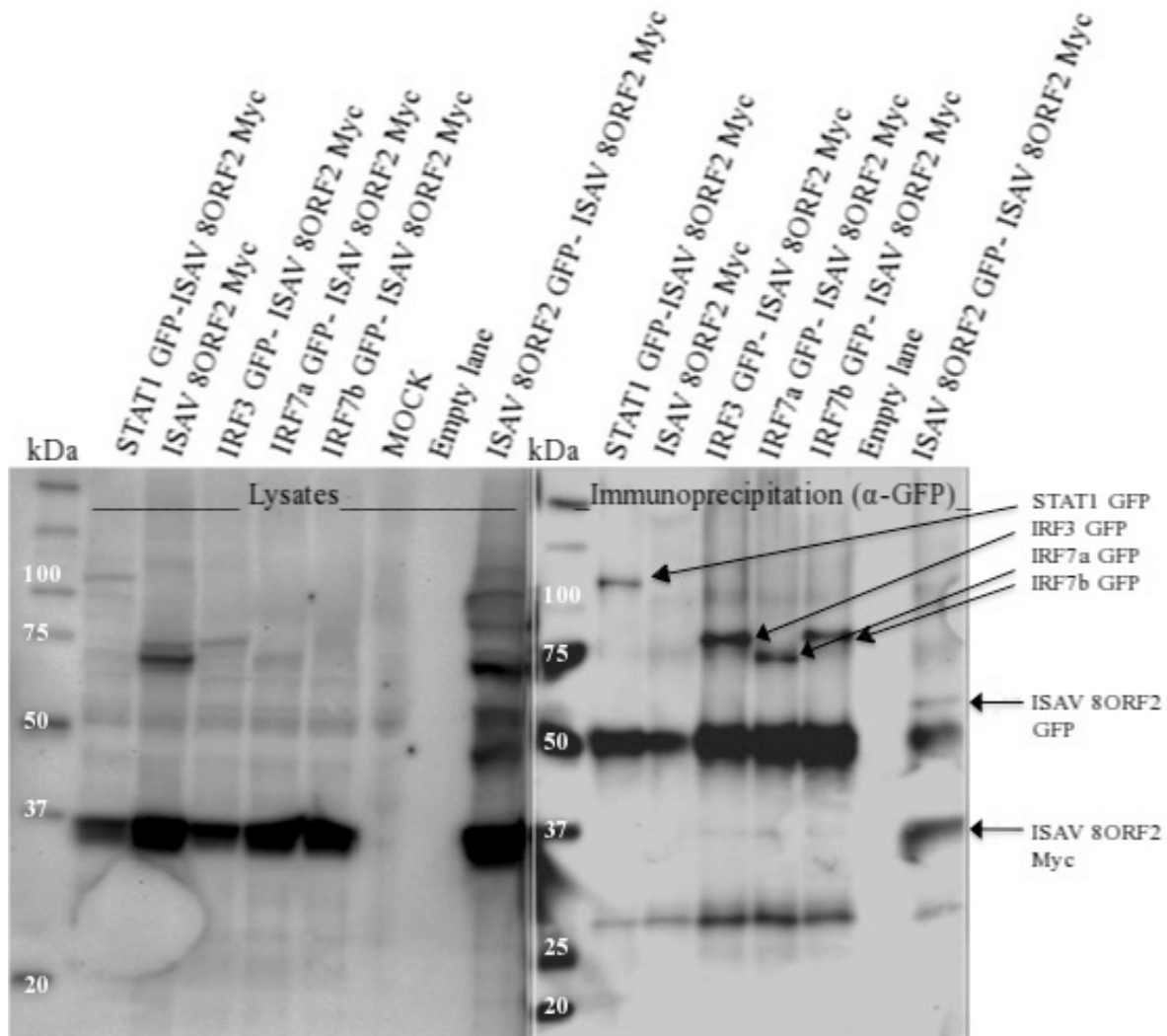


Figure 17b Western Blot of co-IP precipitated with α GFP now incubated with α GFP (in addition to the earlier α Myc incubation in 17a). Here visualizing the GFP tag of precipitated proteins, to see that precipitation occurred.

To validate that co-precipitation had occurred the immunoprecipitations were performed with α GFP pull-down. The incubation was performed with primary and secondary α GFP, as the incubation with α GFP antibodies detects GFP tagged proteins. The molecular size of the GFP tag itself is 30kDa. The results showed that the negative control (STAT1 GFP- ISAV 8ORF2) had a STAT1 GFP band at the size of 115kDa, estimated to 85kDa. The other negative control (ISAV 8ORF2) had no band as predicted. The result of the positive control (ISAV 8ORF2 GFP – ISAV 8ORF2 Myc), showed an ISAV 8ORF2 GFP band at 58kDa, estimated to 28kDa. The results for the interactions found in Y2H are presented as follows: The (IRF3

GFP – ISAV 8ORF2) had an IRF3 GFP band at 80kDa, estimated to 50kDa. The (IRF7a GFP – ISAV 8ORF2) had an IRF7a GFP band at 75kDa, estimated to 45kDa. To validate the estimated molecular sizes, we compared them to their predicted molecular sizes (Appendix 1). The results showed that the proteins were of suitable size.

The findings of the Y2H screening and the further investigation by co-IP are gathered in a schematic map of interactions between key proteins of the IFN system and proteins of the ISAV, shown in figure 18.

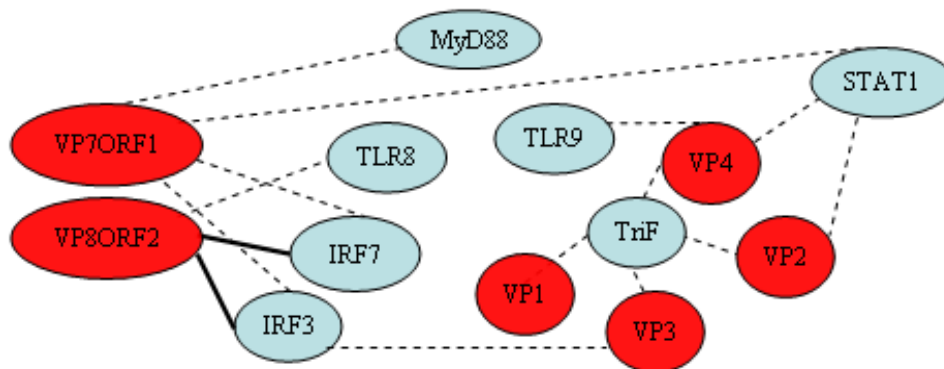


Figure 18: Protein interactions found using yeast-two-hybrid are marked in stippled lines and protein interactions verified with immunoprecipitation are marked in bold lines between the protein symbols. ISAV viral proteins (VP) are marked red, while proteins expressed from Atlantic salmon innate immunity are marked blue.

5.3 Functional studies

5.3.1 Coiled-coil prediction

For the prediction of possible coiled-coil motifs for the Atlantic salmon IRF3 and IRF7a we performed online algorithm programs, Multicoil and Paircoil (for further reference, see section 3.7). The results showed no coiled-coil motifs for any of the IRFs.

5.3.2 Conserved regions within the IRF genes

To investigate whether the IRF proteins of ISAV and human IRF3 contained conserved regions, hypothesized to be important for function, the homology between human- and ISAV-IRF3 and IRF7a was analysed. The results are shown in figure 19.

gi|38492947|pdb|1QWT|B Chain B, Auto-Inhibitory Interferon Regulation Factor-3 (Irf3) Transactivation Domain

gi|219972763|gb|FJ517643.1| *Salmo salar* interferon regulatory factor 3 (IRF3) mRNA, complete cds

gi|159792915|gb|EU153263.1| *Salmo salar* interferon regulatory factor 7 mRNA, complete cds

```

-----SPSLDNPTFPNLPSENPLKRLLVPGEE-WEFEVTAFYRGRQVFQQTISCEPEGLRLV
1 VMEDAVGGAVLP-PQPVYPMDGP--VGAHEQQVVEQFTNELSRTMVGENFKTHFRVSVYYRGLKMLEQLVENEAGFRLV
1 PP-----VLIQQPYAQVNPDALLNLPATRSSLWLEITISYRGSEMLKTQVSGPR-VQLH

-GSEVGDRTLPGWP----VTLDPDGMSLTDRGVMSYVRHVLSCLGGGLALWRAGQWLWAQRLGHCHTYWAVSEELLPNSG
YRPELTQPLLDPSGLNLVSLPSP--PVRDETQAKLTQDILALLGEGLEV GASGPIIYGLRKGGIKAFWSLDK--FDKSR
YQCNALPNTQP-----LCFPSTD-GLPDLKQIEYTNCLILRSVQRGLLLEVQNTGIYGYRQDKCHFVSSTSN---PREA

HGPD-GEVPKDKEGGVFDLGPFIVDLITFTTEGSGRS-PRYALWFCVGESWPQ--DQPWTKRLVMVKVVPTCLRALVEMAR
RPQAVSKFPE----PLYQAKDFYGGLLKFMDSHRGECPPFSLFFCLGEKWPDPDNRPEKKLVMVEVVLTALELLKSIIV
HPEP-RKMPQNEVQLLNFOQYENELIAFKENRRGS-PDYTIHMCFGKFPD--GKPPEKKLIVVKVPLICRYFHEVAQ

VGGASLENT-VDLHISNSHPLSLTSDQYKAYLQDLVEGMDFQGPGES 255
EGGASSLQS--VELQLSLQESLQEVMDLC----- 481
EEGASSLQND-ISLQISHHNSLMELINATWPDGPQHTMGQYF----- 415

```

Figure 19. A protein-BLAST (BLASTp) alignment with human IRF3 as query aligned with IRF3, IRF7a, performed at PubMed.

Chapter 6. Discussion

The type I interferon system is a key player in the host cells inhibition of viral replication during an infection. The ISAV segment 8ORF2 and 7ORF1 have been shown to exert interferon antagonistic activity in Atlantic salmon (García-Rosado, et al., 2008). Knowledge gained by collaborative researchers at UIT and NVI served as a starting point of the practical work in this project. They have previously characterized proteins from the IFN system in Atlantic salmon as well as proteins from the ISAV. In this study, we found several protein-protein interactions between virus and host, these findings are here discussed in the light of known mechanisms in other Orthomyxoviruses with IFN antagonistic genes.

6.1 Screening for protein-protein interactions with Y2H

The protein interactions found by the Y2H screening were divided into strong interactions and weaker interactions. Strongly interacting proteins were indicated by growth on the most selective QDO medium, we found that (IRF3 - ISAV 8ORF2) and (IRF7a - ISAV 8ORF2) interacted strongly. This analysis was confirmed by the positive control (ISAV 8ORF2 - ISAV 8ORF2), which showed massive growth on QDO media, see figure 12. These two strong interactions of (ISAV 8ORF2 - IRF3) and (ISAV 8ORF2 - IRF7a) are both involved in the IFN system and are therefore particularly interesting for IFN antagonistic activity. This will be discussed further in section 6.2 and 6.3.

Growth on the TDO medium indicated a weaker protein-protein interaction, which was found for the pathogen recognition receptors, TLR 8 and TLR 9, both of these TLRs showed interactions with the ISAV, (TLR8 - 8ORF2) and (TLR9 - ISAV segment 4). However, TLR9 is referred to as able to recognize DNA virus and TLR8 to recognize ssRNA virus, making the TLR8 interaction with 8ORF2 more likely because ISAV is an ssRNA virus. The finding of TLR8 interacting with 8ORF2 indicates an IFN induction through the MyD88 signalling pathway. Another interaction of the MyD88 pathway was the interaction between (ISAV

7ORF1 - MyD88), suggesting that this might be the protein-protein interaction that inhibits the IFN production and therefore also inhibit the antiviral state upon viral infection (figure 3.). Dr. J.Ramsell, NVI, earlier found the interaction of MyD88 with ISAV segment 7ORF1. Nonetheless, MyD88 does not necessarily have a key role in the down regulation of IFN, as the MyD88 adaptor primarily induces an interleukin expression (Leichtle, et al., 2009). However, the interactions of TRIF with ISAV segments 1,2,3 and 4 indicate an inhibition of the IFN through the other IFN pathway with TRIF as adaptor molecule (figure 4.). The TRIF is strictly activated by dsRNA virus recognition of TLR3, now TLR 3 were not investigated in this study but have been found for the recognition of ssRNA Influenza A virus (Le Goffic, et al., 2006). A very interesting interaction in mammals involved in the IFN antagonistic activity is the cytoplasmic interaction between viral ssRNA and IRF7a (Haller, et al., 2006). Protein-protein interaction data found in this study supports the data from Haller et al., 2006, where the interaction of (IRF7a - ISAV 7ORF1) was found. The ISAV 7ORF1 has been located in the cytoplasm and is hypothesized as an IFN antagonist (García-Rosado, et al., 2008), indicating an actual antagonistic interaction between the ISAV 7ORF1 and the innate immunity of Atlantic salmon. Due to lack of time, the interactions of (IRF7a - ISAV 7ORF1) were not verified with co-IP in this study.

The non-interacting ISAV proteins 7ORF2 and 8ORF1 both validated with visible protein expressions in Western blot (figure 11a and 11b) suggest that neither the ISAV 7ORF2, nor the ISAV 8ORF1 proteins are responsible for inhibiting the antiviral state. This might be explained by their maintaining functions of the virus (O'Neill, et al., 1998), and (Falk, et al., 2004).

Despite the earlier findings, we failed to validate some of the protein expressions from Y2H vectors; the ISAV segment 2 in pGBD, ISAV segment 1 in pGAD, and ISAV segment 5 and 6 in both pGAD and pGBD (figure 11a and 11b). The results regarding ISAV segment 5 and 6 can be explained by the fact that these vector inserts encodes trans membrane proteins, fusion and hemagglutinin-esterase proteins respectively. Trans membrane proteins have two hydrophilic ends and a lipophilic core expressed over the plasma membrane of the cell, and their expressed protein is therefore hard to analyse (Phizicky & Fields, 1995). The ISAV segment 2 had a insufficient vector insert (Appendix 2) resulting in absent protein expression while ISAV segment 1 in the pGAD vector showed several interactions during Y2H screening, suggesting that a moderate amount of the protein was expressed.

6.2 Verification of protein-protein interaction using co-immunoprecipitation (co-IP)

The co-immunoprecipitation gave results difficult to interpret even though the co-precipitation controls showed the expected results for all co-precipitated samples (figure 17b). We found a 37kDa band of ISAV 8ORF2 Myc (figure 17a), indicating an interactions between (IRF3 GFP – ISAV 8ORF2 Myc) and between (IRF7a GFP – ISAV 8ORF2 Myc), but compared to the positive control, the detection of the interactions was very weak, see figure 17a. This suggests that the interactions between (IRF3 GFP – ISAV 8ORF2 Myc) and (IRF7a GFP – ISAV 8ORF2) were doubtful and that the interactions of them found in Y2H was not detected in co-IP since co-IP analysis is a high confidence assay, which display that Y2H is known to provide false positives (Deane, et al., 2002). However, the IRF3 GFP and IRF7a GFP inserts transfected into HEK293T cells expressed their proteins in moderate amounts. They were both detected with a low amount of fluorophors from their GFP tags (figure16), indicating low transfection efficiency. Even though a 2x and a 5x amount of transfected cells were used for IRF3 GFP and IRF7a GFP, respectively, they were not close of the flourophore intensity of the positive control ISAV 8ORF2 GFP. This indicates that the interaction between (IRF3 GFP – ISAV 8ORF2), and (IRF7a GFP – ISAV 8ORF2) is an actual interaction but with a low transfection efficiency. However, the weak interaction may also depend on the fact that the IRF3 is more accessible for interactions after viral infection, causing a configuration switch of the IRFs (Dragan, et al., 2007). This is further discussed in section 6.3.

To validate the co-IP, all co-transfected samples had an incorporated GFP tag in a vector insert and a Myc tag in the other vector insert. After expression in the host these protein tags were detected with anti GFP antibody (figure 17b), and anti Myc antibody (figure 17a). The interacting proteins were proven by detection of both of their protein GFP and Myc tags. In this study, the co-precipitated negative control (STAT1 GFP – ISAV 8ORF2) showed the co-precipitated GFP protein, while the single-transfected negative control (ISAV 8ORF2 Myc) showed no GFP protein as expected. These results of the negative controls show that no interaction of co-precipitated 8ORF2 Myc was made with the GFP tag alone, and that no false

negatives were produced by single-precipitation of ISAV 8ORF2 Myc proteins, therefore our findings are reliable and not a product of non-specific bindings, which are known as a common interference in co-IP (Phizicky & Fields., 1995). Another aspect of producing false positive results is the possibility to detect interactions between proteins that are never present in the same cell. As the mechanisms of the ISAV are not yet fully understood, it is yet to prove, whether the protein interaction found in this study in fact are present in the same cell.

6.3 Protein-protein interactions and their putative function in the immune system.

ISAV 8ORF2 has a predicted coiled-coil motif (García-Rosado, et al., 2008) and it is stated that protein-protein interactions are mediated by coiled-coil motifs (Huang, et al., 2005). This suggest that the interactions between the proteins of the positive control used in Y2H and co-IP (ISAV 8ORF2 – ISAV 8ORF2), mediate dimerization of their proteins through their coiled coil motifs. This indicates that the IRF3/IRF7a interaction with ISAV 8ORF2 can be a result of coiled-coil mediated interaction between the residues. Nonetheless, IRF3 and IRF7a were not detected with coiled-coil motifs using the coiled-coil prediction programs Paircoil and Multicoil. However, the IRF3/IRF7a were found to interact with ISAV 8ORF2, which has been located to the nucleus (García-Rosado, et al., 2008). This suggests a likely IFN antagonistic protein-protein interaction in the nucleus of ISAV 8ORF2 and IRF3, as IRF3 is located to the cell nucleus upon viral infection (figure3.) and a inducer of IFN that provides the antiviral state in the host cell (Robertsen, 2006). The result from the coiled-coil prediction studies in this thesis suggests that the interaction between ISAV 8ORF2 and IRF3 is not a coiled-coil mediated interaction. However, IRF3 in mammals is thought to be activated first upon virus infection by phosphorylation's in the C-terminal, that increases the negative charge of IRF3 that results in the IRF-3 dimerization (Dragan, et al., 2007). This suggests that Atlantic salmon IRF3 and IRF7a make the folding of coiled-coil first post virus infection. Moreover, the IRF3 and IRF7a transfection with ISAV 8ORF2 is performed with non-infected cells, suggesting a low, if any, activation of the essential coiled coil motif of IRF3 to induce IFN (Dragan, et al., 2007). Furthermore the IRF3 and the IRF7a are suggested to be activated the same way (Iwamura, 2001). Those findings are shown to be likely in this thesis, where the conserved regions important for function are shown to be very similar for IRF3 and IRF7a (figure 19). Moreover, the evolutionary-conserved regions for human IRF3 show great similarity to the IRF3 and IRF7a proteins from Atlantic salmon (figure19), indicating that the

function of human IRF3 equals the Atlantic salmon IRFs. The phosphorylation activation of IRF3 has been mimicked by phosphomimetic Asp substitution of Ser/Thr mutagenesis *in vitro* in Human, demonstrating the full coiled-coil motif of IRF3 first after activation (Dragan, et al., 2007). If the functions of Human IRF3 and Atlantic salmon IRF3 are the same, the assumption can be made that the interaction between the IRFs and ISAV 8ORF2 proteins in this study would have been stronger if both of the IRF were investigated with ISAV stimulated cells.

Moreover, Orthomyxoviruses have evolved from the same origin and Influenza A, Thogovirus, and ISAV all have strategies to suppress the induction of type I IFNs. Thus indicating that the mechanisms to suppress the IFN induction could be similar among Orthomyxoviruses. It has earlier been shown that not only one gene is responsible for IFN antagonism of ISAV, as common for Thogovirus and Influenza virus (Jennings, et al., 2005), (Talon, et al., 2000), but both ISAV 7ORF1 and 8ORF2 genes suppress IFN independent of each other (García-Rosado, et al., 2008). The NS1 of Influenza A is suggested to suppress IFN through the IRF3 inhibition in the cytoplasm (Talon, et al., 2000), a feature that is shared with the ISAV segment 7ORF1. This segment is also located in the cytoplasm with hypothesized IFN antagonistic activity, suggesting that the ISAV 7ORF1 interaction with IRF3/IRF7a in the cytoplasm is involved in the ISAV antagonistic activity through suppression of the IFN positive feedback (Haller, et al., 2006). However, the ISAV 7ORF1 interaction with IRF3/IRF7a could not be analysed with co-IP due to the lack of time, and therefore would have to be verified in further studies. The hypothesized IFN suppression of ISAV 8ORF2 localized in the nucleus (García-Rosado, et al., 2008) has similar characteristics as the IFN antagonistic ML protein of Thogovirus, inhibiting IFN transcription in the nucleus (Jennings, et al., 2005). Furthermore the ISAVs two IFN antagonistic proteins encoded by segment 7ORF1 and 8ORF2, are hypothesized to be independent of each other (García-Rosado, et al., 2008). This suggests that they do not interact with the same IRF in native form, but that ISAV 8ORF interacts with IRF3, as both are localized in the nucleus, and ISAV 7ORF1 interacts with IRF7a, as both are localized in the cytoplasm.

6.4 Further aspects

To get a better understanding of the protein-protein interactions of (ISAV 7ORF1 - IRF7a) and (ISAV 8ORF2 – IRF3), we need further studies for verification, in order to get a clearer picture of the mechanisms involved during IFN antagonism caused by ISAV. To enable a more distinct detection during co-IP studies we could use a huge amount of co-transfected host cells, or as a second opportunity to study viral activated IRFs.

The mechanisms of the IFN system upon viral infection are not yet fully understood, and more research is needed to get one step closer to a fully understanding of the ISAV host mechanisms. Which in the end might be used in the development of more efficient prevention methods such as a vaccine against ISAV, or for other virus within the *Orthomyxoviridae*.

Chapter 7. Literature

- Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, 215(3), 403–410.
- Aspehaug, V., Mikalsen, A.B., Snow, M., Biering, E., and Villoing, S. (2005). Characterization of the Infectious Salmon Anemia Virus Fusion Protein. *Journal of Virology*, 79(19), 12544-12553.
- Asphaug, V., Falk, K., Krossøy, B., Thevarajan, J., Sanders, L., Moore, L., Endresen, . and Biering, E. (2004). Infectious salmon anemia virus (ISAV) genomic segment 3 encodes the viral nucleoprotein (NP), an RNA-binding protein with two monopartite nuclear localization signals (NLS). *Virus Research*, 106(1), 51-60.
- Berg, K., Svingerud, T., Sun, B., and Robertsen, B. (2009). An antiserum against Atlantic salmon IFN α 1 detects IFN and neutralizes antiviral activity produced by poly I:C stimulated cells. *Developmental & Comparative Immunology*, 33(4), 638-645.
- Bergan, V., Kileng, Ø., Sun, B., and Robertsen, B. (2010). Regulation and function of interferon regulatory factors of Atlantic salmon. *Molecular Immunology*, 47(11-12), 2005-2014.
- Berger, B., Wilson, D.B., Wolf, E., Tonchev, T., Milla, and Kim, P.S. (1995). "Predicting Coiled Coils by Use of Pairwise Residue Correlations". *Proceedings of the National Academy of Science USA*, 92, 8259-8263.
- Biering, E., Falk, K., and Hoel, E. (2002). Segment 8 encodes a structural protein of infectious salmon anemia virus (ISAV): the co-linear transcript from segment 7 probably encodes a non-structural or minor structural protein. *Diseases of aquatic organisms* 49(2), 117-122.
- Borden, E. C., Sen, G.C., Uze, G., Silverman, R.H., Ransohoff, R.M., Foster, G.R., Stark, and G.R. (2007). Interferons at age 50: past, current and future impact on biomedicine. *Nature Reviews Drug Discovery*, 6(12), 975-990.
- Cramer, A., Whitehorn, E. A., Tate, E., and Stemmer, W. P. (1996). Improved Green Fluorescent Protein by Molecular Evolution Using DNA Shuffling. *Nature Biotechnology* 14, 315-319.
- Dannevig, B.H., Falk, K., and Namork, E. (1995). Isolation of the causal virus of infectious salmon anaemia (ISA) in a long-term cell line from Atlantic salmon head kidney. *Journal of General Virology*, 76(6), 1353-1359.
-

-
- Deane, C. M., Salwiński, L., Xenarios, I., and Eisenberg, D. (2002). Protein Interactions: Two Methods for Assessment of the Reliability of High Throughput Observations. *Molecular & Cellular Proteomics*, 1(5), 349–356.
- Dragan, A., Hargreaves, V.V., Makeyeva, EN., and Privalov, PL. (2007). Mechanisms of activation of interferon regulator factor 3: the role of C-terminal domain phosphorylation in IRF-3 dimerization and DNA binding. *Nucleic Acids Research*, 35(11), 3525-3534.
- Estojak, J., Brent, R. and Golemis, E.A. (1995). Correlation of two-hybrid affinity data with in vitro measurements. *Molecular and Cellular Biology*, 15 (10), 5820-5829.
- Falk, K., Aspehaug., Vlasak. R., and Endresen, C. (2004). Identification and Characterization of Viral Structural Proteins of Infectious Salmon Anemia Virus. *Journal of Virology*, 78(6), 3063–3071.
- Fields. S., and Song, O. (1989). A novel genetic system to detect protein-protein interactions. *Nature*, 340, 245–247.
- García-Rosado, E., Markussen, T., Kileng, Ø., Bækkevold, E. S., Robertsen, B., and Mjaaland, S., (2008). Molecular and functional characterization of two infectious salmon anaemia virus (ISAV) proteins with type I interferon antagonizing activity. *Virus Research*, 133(2), 228-238.
- Garcia-Sastre, A., and Biron, C.A. (2006). Type 1 interferons and the virus-host relationship: A lesson in detente. *Science*, 312(5775), 879-882.
- Gasteiger, E., Gattiker, A., Hoogland, C., Ivanyi, I., Appel, R. D., and Bairoch, A. (2003). ExPASy: The proteomics server for in-depth protein knowledge and analysis. *Nucleic acids research*, 31(13), 3784-3788.
- Grimholt, U., Larsen, S., Nordmo, R., Midtlyng, P., Kjoeglum, S., and Storset, A., (2003). MHC polymorphism and disease resistance in Atlantic salmon (*Salmo salar*); facing pathogens with single expressed major histocompatibility class I and class II loci. *Immunogenetics*, 55(4), 210-219.
- Hacker, H., Redecke, V., and Blagoev, B. (2006). Specificity in Toll-like receptor signalling through distinct effec- tor functions of TRAF3 and TRAF6. *Nature*, 439, 204–207.
- Hagmaier, K., Jennings, S., and Buse, J. (2003). Novel gene product of Thogoto virus segment 6 codes for an interferon antagonist. *Journal of Virology*, 77(4), 2747-2752.
- Haller, O., Kochs, G., and Weber, F. (2006). The interferon response circuit: Induction and suppression by pathogenic viruses. *Virology*, 344(1), 119-130.
- Haller, O., Stertz., and Kochs, G. (2007). The Mx GTPase family of interferon-induced antiviral protein. *Microbes and infection*, 9, 1636-1643.
- Harrison, S.C. (2005). Mechanism of membrane fusion by viral envelope proteins. *Advances in Virus Research*, 64, 231–261.
-

-
- Hellebo, A. Vilas, U., and Falk, K. (2004). Infectious salmon anemia virus specifically binds to and hydrolyzes 4-O-acetylated sialic acids. *Journal of Virology*, 78 (6), 3055-3062
- Hetland, D.L., Jørgensen, S.M., Skjødt, K., Dale, O.B., Falk, K., Xu, C., Mikalsen, A.B., Grimholt, U., and Gjøen, T. (2010). In situ localisation of major histocompatibility complex class I and class II and CD8 positive cells in infectious salmon anaemia virus (ISAV)-infected Atlantic salmon. *Fish and Shellfish Immunology*, (28), 30–39.
- Honda, K., Yanai, H., Negishi, H., Asagiri, M., Sato, M., Mizutani, T., Shimada, N., Ohba, Y., Takaoka, A., Yoshida, N., and Taniguchi, T. (2005). IRF-7 is the master regulator of type-I interferon-dependent immune responses. *Nature*, 434(7034), 772–777.
- Iwamura, T., Yoneyama, M., Yamaguchi, K., Suhara, W., Mori, W., Shiota, K., Okabe, Y., Namiki, H., and Fujita, T. (2001). Induction of IRF-3/-7 kinase and NF-kappaB in response to double-stranded RNA and virus infection: common and unique pathways. *Genes Cells*, 6(4), 375–388.
- James, P., Halladay, j., and Craig, E.A. (1996). Genomic Libraries and a Host Strain Designed for Highly Efficient Two-Hybrid Selection in Yeast. *Genetics*, 144(4), 1425–1436.
- Jennings, S., Martinez-Sobrido, L., and Garcia-Sastre, A. (2005). Thogoto virus ML protein suppresses IRF3 function. *Virology*, 331(1), 63-72.
- Katze, M.G., Yipeng, H., Y. and Gale, M. (2002). Viruses and interferon: a fight for supremacy. *Nature Review Immunology*, 2(9), 675-687.
- Kibenge, F., Godoy, M., Wang, Y., Kibenge, M., Gherardelli, V., Mansilla, S., Lisperger, A., Jarpa, M., Larroquete, G., Avendaño, F., Lara, M., and Gallardo, A. (2009). Infectious salmon anaemia virus (ISAV) isolated from the ISA disease outbreaks in Chile diverged from ISAV isolates from Norway around 1996 and was disseminated around 2005, based on surface glycoprotein gene sequences. *Virology Journal*, 6(1), 1-16.
- Kileng, Ø., Albuquerque, A., and Robertsen, B. (2008). Induction of interferon system genes in Atlantic salmon by the imidazoquinoline S-27609, a ligand for Toll-like receptor 7. *Fish & Shellfish Immunology*, 24(5), 514-522.
- Kileng, Ø., Bergan, V., Workenhe, S.T., and Robertsen, B. (2009). Structural and functional studies of an IRF-7-like gene from Atlantic salmon. *Developmental & Comparative Immunology*, 33(1), 18-27.
- Knipe, D.M., and Howley, P, M. (2007). *Fields Virology* (fifth ed.): Lippincottt, Williams & Wilkins.
- Landy, A. (1989). Dynamic, Structural, and Regulatory Aspects of Lambda Site-specific Recombination. *Annual Review of Biochemistry*, 58, 913-949.
- Le Goffic, R., Balloy, V., Lagranderie, M., Alexopoulou, L., Escriou, N., Flavell, R., Chignard, M., and Si-Tahar, M. (2006). Detrimental contribution of the Toll-like
-

-
- receptor (TLR)3 to influenza A virus-induced acute pneumonia. *Public Library of Science Pathogens*, 2(53).
- Leichtle, A., Hernandez, M., Pak, K., Webster, N.J., Wasserman, S.I., and Ryan A.F. (2009). The toll-Like receptor adaptor TRIF contributes to otitis media pathogenesis and recovery. *BioMed central Immunology*, 10(45).
- Markussen, T., Jonassen, C.M., Numanovic, S., Braaen, S., Hjortaas, M., and Nilsen, H. (2008). Evolutionary mechanisms involved in the virulence of infectious salmon anaemia virus (ISAV), a piscine orthomyxovirus. *Virology*, 374(2), 515-527.
- McBeath, A.J., Collet, B., Paley, R., Duraffour, S., Aspehaug, V., Biering, E., Secombes, C.J and Snow, M. (2006). Identification of an interferon antagonist protein encoded by segment 7 of infectious salmon anaemia virus. *Virus Research*, 115(2), 176-184.
- Mjaaland, S., Rimstad, E., Falk, K., and 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(10), 7681-7686.
- O'Neill, R., E., Talon, J., and Palese, P. (1998). The influenza virus NEP (NS2 protein) mediates the nuclear export of viral ribonucleoproteins. *The EMBO Journal* 17, 288-296
- Palese, P. (1977). The genes of influenza virus. *Cell*, 10(1), 1-10.
- Pedersen, T., Skjesol, A., and Jorgensen, J.B. (2007). VP3, a structural protein of infectious pancreatic necrosis virus, interacts with RNA-dependent RNA polymerase VP1 and with double-stranded RNA. *Journal of virology*, 81(12), 6652-6663.
- Phizicky, E.M. and Fields, S. (1995). Protein-protein interactions: Methods for detection and analysis. *Microbiological Reviews*, 59, 94-123.
- Press, C.M and Evensen, O. (1999). The morphology of the immune system in teleost fishes. *Fish & Shellfish Immunology*, 9, 309-318.
- Qin, B., Liu, C., Lam, S.S., Srinath, H., Delston, R., Correia, J.J., Derynck, R., and Lin, K. (2003). Crystal structure of IRF-3 reveals mechanism of autoinhibition and virus-induced phosphoactivation. *Nature Structural Biology*, 10(11), 913-921.
- Robertsen, B. (2011). Can we get the upper hand on viral diseases in aquaculture of Atlantic salmon? *Aquaculture Research*, 42, 125-131.
- Robertsen, B. (2006). The interferon system of teleost fish. *Fish & Shellfish Immunology*, 20(2), 172-191.
- Sambrook, J. and Russel, D.W. (2001). *Molecular Cloning A laboratory manual*, 3rd edition. Cold: Cold Spring Harbour Laboratory Press.
- Samuel, C.E. (2001). Antiviral actions of interferons. *Clinical Microbiology Review*, 14(4), 778-809.
-

-
- Skehel, J.J., and Wiley, D.C. (2000). Receptor binding and membrane fusion in virus entry: The Influenza Hemagglutinin Annual *Review of Biochemistry*, 69, 531-569.
- Skjesol, A., Hansen, T., Shi, C.Y., Thim, H.L. and Jørgensen, J.B. (2009). Structural and functional studies of the STAT1 gene from Atlantic salmon. *Unpublished*.
- Skjæveland, I., Iliev, D.B., Strandskog, G., and Jørgensen, J.B. (2009). Identification and characterization of TLR8 and MyD88 homologs in Atlantic salmon (*Salmo salar*). *Developmental & Comparative Immunology*, 33(9), 1011-1017.
- Skjæveland, I., Iliev, D.B., Zou, J., Jørgensen, T., and Jørgensen, J.B. (2008). A TLR9 homolog that is up-regulated by IFN- γ in Atlantic salmon (*Salmo salar*). *Developmental & Comparative Immunology*, 32(6), 603-607.
- Snow, M., Ritchie, R., Arnaud, O., Villoing, S., Aspehaug, V., and Cunningham, C.O. (2003). Isolation and characterisation of segment 1 of the infectious salmon anaemia virus genome. *Virus Research*, 92(1), 99-105.
- Solar, I.I. (2009). Use and exchange of salmonid genetic resources relevant for food and aquaculture. *Reviews in Aquaculture*, (1), 174–196.
- Spring, K.R. and Davidson, M.W. (2010). Introduction to Fluorescence Microscopy. <http://www.microscopyu.com/articles/fluorescence/fluorescenceintro.html>
- Stetson, D.B., Medzhitov, R. (2006). Type I interferons in host defense. *Immunity* 25, 373–338.
- Talon, J., Horvath, C.M., and Polley, R. (2000). Activation of interferon regulatory factor 3 is inhibited by the influenza A virus NS1 protein. *Journal of Virology*, 74(17), 7989-7996.
- Thorud, K.E., and Djupvik, H.O. (1988). Infectious salmon anaemia in Atlantic salmon (*Salmo salar* L). *European Association of Fish Pathologist*, 8, 109–111.
- Vågsholm, I., Djupvik, H.O., Willumsen, F.V., Tveit, A.M., and Tangen, K. (1994). Infectious salmon anaemia (ISA) epidemiology in Norway. *Preventive Veterinary Medicine*, 19(3-4), 277-290.
- Weaver, K.E., Kwong, S.M., Firth, N., and Francia, M.V. (2009). The RepA_N replicons of Gram-positive bacteria: A family of broadly distributed but narrow host range plasmids. *Plasmid*, 61(2), 94-109.
- William, P. (2008). Fundamental Immunology. *Sixth edition*, chapter 23.
- Wolf, E., Kim, P.S., and Berger, B. (1997). MultiCoil: a program for predicting two- and three-stranded coiled coils. *Protein science: a publication of the Protein Society*, 6(6), 1179-1189.
-

Appendix 1 Predicted molecular size of expressed pDEST inserts

Predicted molecular size of expressed pDEST insert

<i>Insert</i>	<i>Predicted (kDa)</i>
S1-PB2	79
S2-PB1	80
S3-NP	66-71/ 77
S4-PA	65
S5-F	50-53/ 45
S6-HE	37/ 42-43
S7O1	34
S7O2	16
S8O1	22-24
S8O2	27/ 35
IRF3	53
IRF7a	48
IRF7b	52
STAT-1	87

Predicted molecular size (kDa) performed by Expasy.

Appendix 2 Sequence of pGBD 2 expression vector for insert

