Faculty of Biosciences, Fisheries and Economics

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Studies of JAK/STAT signaling in Atlantic salmon

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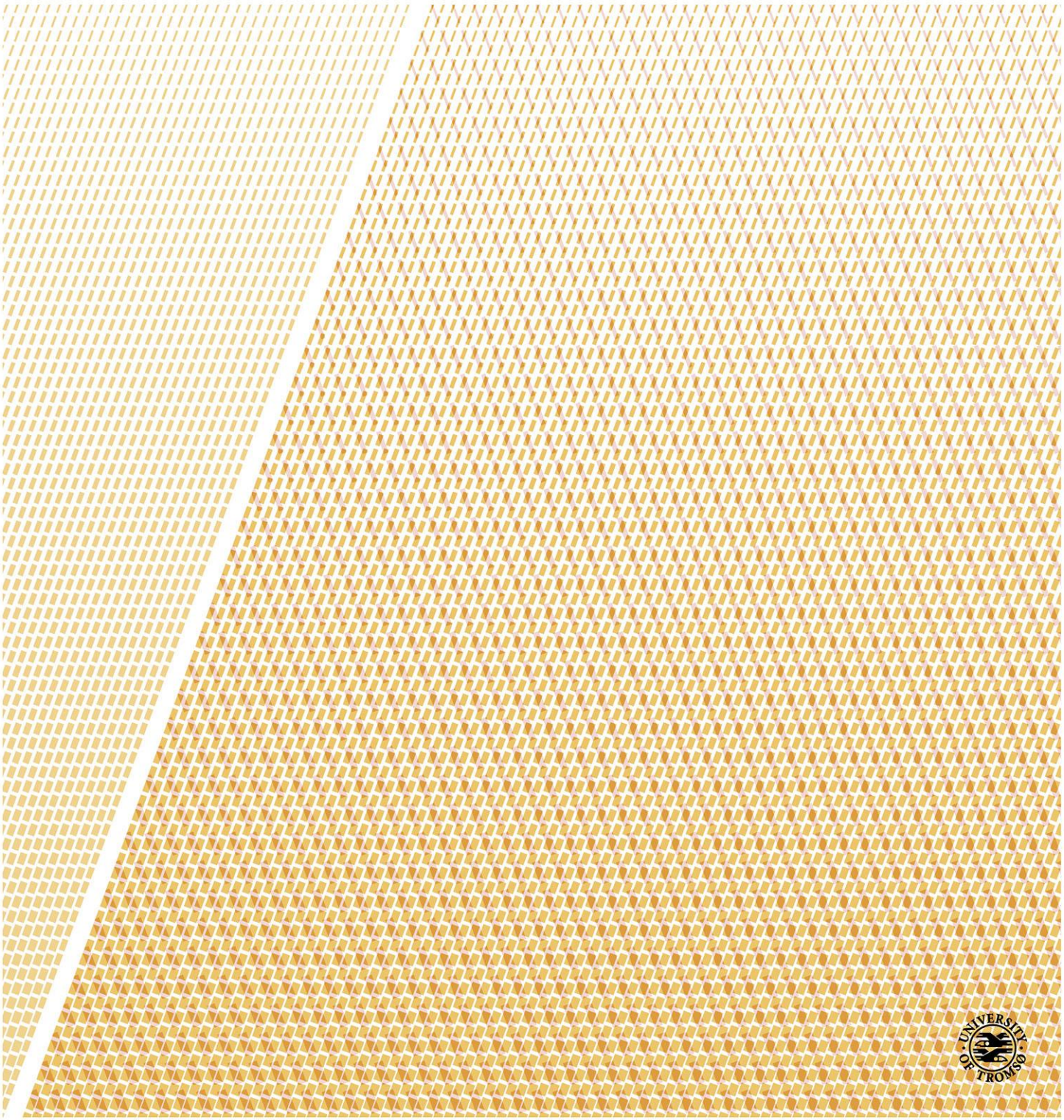

Acknowledgements

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Contents

Abbreviations

aa	Amino acid
APC	Antigen presenting cells
ATP	Adenosine triphosphate
CHSE-214	Chinook salmon embryo cells
CISH	Cytokine-inducible SH2- containing protein
CpG	Cytosine-phosphate-guanine
DBD	DNA-binding domain
DNA	Deoxyribonucleic acid
FERM	Four point, ezrin, radixin, moesin
GAS	Gamma interferon activation site
HK	Head kidney
IFN	Interferon
IFNAR	Interferon α/β receptor
IFNGR	Interferon γ receptor
IL28R	Interleukin 28 receptor
IL-10R2	Interleukin 10 receptor 2
IPNV	Infectious pancreatic necrosis virus
IRF	Interferon regulatory factor
ISAV	Infectious salmon anemia virus
ISG	Interferon stimulated gene
ISGF3	Interferon stimulated gene factor 3
ISRE	Interferon stimulated response element
JAK	Janus kinase

JH	Janus homology
kDa	Kilo Dalton
KIR	Kinase inhibitor region
LPS	Lipopolysaccharide
mRNA	messenger RNA
Mx	Myxovirus resistance
MyD88	Myeloid differentiation primary response gene 88
NES	Nuclear export signal
ND	N-terminal domain
NK	Natural killer cells
NLR	Nod like receptor
NLS	Nuclear localization signal
PAMP	pathogen-associated molecular pattern
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendrittic cell
PIAS	Protein inhibitor of activated STAT1
Poly I:C	Polyinosinic polycytidylic acid
PRR	Pattern recognition receptor
qPCR	quantitative PCR
PTP	protein tyrosine phosphatases
RIG-I	Retinoic acid inducible gene I
RLR	RIG-I- like receptors
RNA	Ribonucleic acid
SH2	Src homology 2
SHP	SH2-containing Protein Tyrosine Phosphatase

PTP	Protein Tyrosine Phosphatase
SOCS	Supressor of cytokine signaling
STAT	Signal transducers and activators of transcription
TAD	Transcriptional activation domain
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TO	Atlantic salmon head kidney cell-line
Tyk2	Tyrosine kinase2

Summary

In mammals the JAK-STAT pathway plays a significant role in mediating the biological activities of both type I and type II IFN, where JAKs are receptor associated kinases and STATs the transcription factors they activate. At the start of this project the knowledge of the JAK-STAT pathway in fish in general was scarce and there were few reports describing the function of any JAK-STAT family member in the Atlantic salmon. There was also very limited knowledge about molecules involved in regulation of this signaling pathway in fish. In this project period we have cloned and / or initiated studies of the functional activity of the transcription factors STAT1, STAT2 and IRF9 and also the TYK2 kinase, as well as several members of the suppressors of cytokine signaling (SOCS) family derived from salmon.

In paper I two full length and two truncated isoforms of salmon Tyk2 were identified. The full length Tyk2 had the same domain structure as its mammalian counterpart and like its mammalian homolog salmon TYK2 was shown to be autophosporylated on tyrosine residues when overexpressed in salmon cell lines, while a mutation in the ATP binding site abolished this activity. This suggests that the phosphorylation of salmon TYK2 is a result of auto-phosphorylation and not caused by the activity of another kinase. Our experiments showed that cells overexpressing TYK2 produced higher levels of Mx transcripts and Mx-promoter activation upon IFN-treatment then the controls overexpressing empty vector. These results suggest that TYK2 in salmon has a role in mediating IFN-signaling.

In paper II salmon IRF9 and two STAT2 isoforms (STAT2a and STAT2b) were cloned and further characterized and the identified molecules showed functional conservation to their mammalian counterparts. STAT2a and STAT2b were tyrosine phosphorylated as result of both type I and type II IFN stimulation and they translocated to the nucleus when co-expressed with STAT1a or IRF9. STAT1a did interact with STAT2a and STAT2b, and all three molecules did interact with IRF9 as well as Tyk2.

Regulation of innate immune responses, including IFN-induction, is critical since excessive inflammatory reaction can be deleterious. The SOCS proteins are involved in the negative regulation of the JAK/STAT pathway. Although, SOCS proteins have been studied extensively

in mammals they are still poorly characterized in lower vertebrates, including fish. In the third paper in this thesis we have cloned and characterized 3 Atlantic salmon SOCS-family members, SOCS1, SOCS2 and CISH, which all showed high conservation to their mammalian homologs. In the basal state all three SOCS-molecules were expressed at low levels in organs and cells. However, upon stimulation by IFNs or TLR-ligands their expression, and SOCS1 in particular, were rapidly induced, showing that their induction occur through ligands associated with both JAK/STAT and TLR-signaling. The most exciting finding regarding the SOCS-protein was that SOCS1 very potently inhibited both IFNa1 and IFN□ signaling and thus have a putative role in IFN-mediated antiviral responses in fish.

In conclusion, this work contributes to understanding of JAK/STAT signaling in Atlantic salmon and shows that this signaling pathway is conserved between Atlantic salmon and mammals, although there are some differences between them which calls for further investigations.

List of papers

Paper 1

Mehrdad Sobhkhez, Tom Hansen, Dimitar B. Iliev, Astrid Skjesol, Jorunn B. Jørgensen

The Atlantic salmon protein tyrosine kinase Tyk2: molecular cloning, modulation of expression and function. Dev Comp Immunol. 41(4): 553 – 63.

Paper 2

Sobhkhez, M., Skjesol, A., Thomassen E., Greiner–Tollersrud L., Iliev, D.B., Sun, B., Robertsen B. & J. B. Jørgensen (2014)

Structural and functional characterization of salmon STAT1, STAT2 and IRF9 homologs sheds light on IFN signaling in teleosts. Manuscript.

Paper 3

Astrid Skjesol, Theresa Liebe, Dimitar B. Iliev, Ernst I Thomassen, Linn G. Tollersrud, Mehrdad Sobhkhez, Lisbeth Lindenskov Joensen, Chris J. Secombes and Jorunn B. Jørgensen

Functional conservation of suppressors of cytokine signaling proteins between teleosts and mammals: Atlantic salmon SOCS1 binds to JAK/STAT family members and suppresses type I and II IFN signaling. Dev Comp Immunol 45(1): 177 – 89.

Background

Teleost fish species constitute almost half of the vertebrate species in existence today and different members of teleost fish are farmed in greater numbers than ever before. This is a stressful process which also renders fish susceptible to different viral infections. In addition to the negative impact on the fish welfare, infectious outbreaks cause major economic losses which the farming industry has to deal with. The JAK / STAT signaling is a conserved signaling pathway in the animals. In higher vertebrates detection of virus leads to expression of interferons (IFNs) which activates JAK/STAT signaling pathway. Activation of JAK/STAT signaling leads to expression of many ISGs that induce an antiviral state in the organism. This fact inevitably makes this signaling pathway a major target for immune evasion strategies used by viruses. Thus a detailed understanding of molecules involved in the JAK/STAT signaling is an important pre-requisite in the understanding of viruses immune evasion strategies and a necessary step in devising ways to counter them.

Introduction:

The immune system of higher vertebrate is divided into an innate and an adaptive branch. The innate immune system is the first line of defense against the invading microorganisms and viruses, and is immediately available to combat threats. It responds to common structures shared by groups of pathogens, these common structures are called pathogen associated molecular patterns (PAMPs) and they are detected through pathogen recognition receptors (PRRs). The different PRRs are specific for different components of microbes and viruses like LPS, proteins, peptidoglycans, DNA and /or RNA and they are constitutively expressed in effector cells of the innate immune system including professional antigen presenting cells (APC) and epithelial cells (Medzhitov and Janeway, 1997).

Several families of PRRs are recognized including Toll like receptors (TLRs), RIG-I- like receptors (RLRs), NOD-like receptor (NLRs) and cytosolic DNA receptors, reviewed in (Beutler, 2009, O'Neill et al., 2013, Loo and Gale, 2011, Barber, 2011, Kvarnhammar et al.,

2011). While TLRs are expressed either on the cell surface or on lysosomal or endosomal membranes, the RLRs and the NLRs are found in the cytoplasm. Upon binding to their PAMPs, most PRRs activate different signaling pathways that regulate the activation of different transcription factors resulting in the secretion of many cytokines and chemokines from innate immune cells.

Cytokines and the immune system

Cytokines are small signaling proteins / peptides that are secreted by cells which mediate and regulate immunity, inflammation and hematopoiesis. They exert their function through autocrine (binding to the receptors on the cells that secrete them) and/or paracrine (binding to the receptors on secreting cells and those in their proximity) and/or endocrine (entering circulation and effecting cells in other parts of the body) activity.

During viral infections, some of the most important defense molecules produced are IFNs and they were also the first cytokine group identified. IFN was discovered as a secretory factor when Isaacs and Lindenmann were studying an already established principle of virus interference (Isaacs and Lindenmann, 1957). IFNs are cytokines that "interfere" with virus replication; they are produced and secreted by the infected cells of the organism in response to virus infection and act in autocrine and paracrine manner (Gautier et al., 2005, Gao et al., 1998, Curtsinger et al., 2012). Expression of IFNs leads to expression of many IFN stimulated genes (ISGs), among them many antiviral proteins.

In human as well as in a number of other mammalian species there are 3 classes of IFNs identified; type I, type II and type III IFNs, and the 3 different classes are shown to interact with different receptors and to trigger specific signaling pathways. Type I IFN, which in mammals include IFNα`s and IFNβ, are most likely produced by almost all tissues and cell types when they are exposed to appropriate stimuli (Pestka, 2007, Pestka et al., 2004). Type II IFN consists of IFNγ and is predominantly produced by T-cells and NK-cells (Schroder et al., 2004). The main function of IFNγ is probably not being an antiviral cytokine, but rather acting as a regulatory cytokine in both innate and adaptive immunity. Type III IFN include IFNλ`s (Sheppard et al.,

2003). While type I and type II IFN receptors are expressed by most cells, expression of receptors for type III IFN are highly restricted (Sommereyns et al., 2008, Pott et al., 2011). Both type I, II and III IFN demonstrate antiviral activity (Osterlund et al., 2005, Schroder et al., 2004, Kotenko et al., 2003), however, its type I IFN that is involved in the early response to viral infection. Several members of type I IFN with high structural homology are identified in mammals, 13 IFNα (IFNα-1, 2, 4, 5, 6, 7, 8, 10, 13, 14, 16, 17, 21) and one IFNβ and also IFN-ε, κ,τ and more (Pestka et al., 2004). Although most cell types do secrete type I IFN, there are several types of cells in different tissues that express type I IFN in response to various viral infections.

Cells producing type I IFNs

One of the main type I IFN producing cell type is plasmacytoid dendritic cells (pDC`s) reviewed in (McKenna et al., 2005, Reizis et al., 2011). These cells are resistant to viral replication since they constitutively express molecules like IFN regulatory factor (IRF) 7 that are important for type I IFN expression (Izaguirre et al., 2003). pDC`s are activated through the MyD88 signaling pathway. This occurs when viruses enter endosomal compartments through endocytosis or autophagy and their RNA and / or DNA is detected by the endosomal receptors TLR7 and 9, respectively (Lund et al., 2004, Krug et al., 2004, Lund et al., 2003). pDC`s are an important source of type I IFN in systemic infections (Swiecki et al., 2013) and in defense against infections in skin (Bond et al., 2012), but their impact is limited in time and capacity. Some of the other cell types involved in type I IFN secretion are epithelial and alveolar macrophages that are lining the airways and that are the main type I IFN producing cells during mucosal infections (Jewell et al., 2007, Kumagai et al., 2007). Epithelial cells produce type I IFN in gut and lungs in response to retrovirus and influenza virus, respectively, and neurons and astrocytes are an important source of type I IFN during brain infection (Delhaye et al., 2006, Kallfass et al., 2012).

Many cytokines, including the IFNs transmit signals from cell-surface to the nucleus by activating members of the signal transducers and activators (STAT) family. The STAT proteins are activated by tyrosine phosphorylation that alters their confirmation to allow specific binding to other proteins and nuclear localization (Kiu and Nicholson, 2012). Type I IFN receptors

(IFNAR1 and IFNAR2) and type III IFN receptors (IL28R / IL-10R2) are associated with Janus Kinase (JAK) 1 and Tyrosine kinase 2 (Tyk2), while type II IFN receptors (IFNGR1and IFNGR2) are associated with JAK1 and JAK2 (Pestka et al., 2004).

JAK / STAT signaling pathway

The Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway is one of the main pathways used to transduce signals from the cell membrane to the nucleus in animals from human to fruitfly. Principal compounds of JAK / STAT signaling pathway are JAKs and STATs, and in the case of type I IFN activated JAK / STAT signaling, also IRF9. The JAK / STAT signaling pathway is activated when ligands (i.e. different cytokines) bind to the extracellular domain of specific cellular receptors. This interaction leads to dimerization / multimerization of the receptor subunits, which bring JAKs – that are non-covalently associated with the intracellular domains of the receptors – in proximity of each other. This leads to auto- and transactivation of JAKs. Activated JAKs phosphorylate tyrosine residues on the receptors intracellular domains and on their substrates, the STATs. Phosphorylation of STATs leads to dimerization and translocation to the nucleus where they bind to specific DNA sequences and induce transcription of their target genes (Kiu and Nicholson, 2012, Stark and Darnell, 2012, Heim, 1999) (Figure 1).

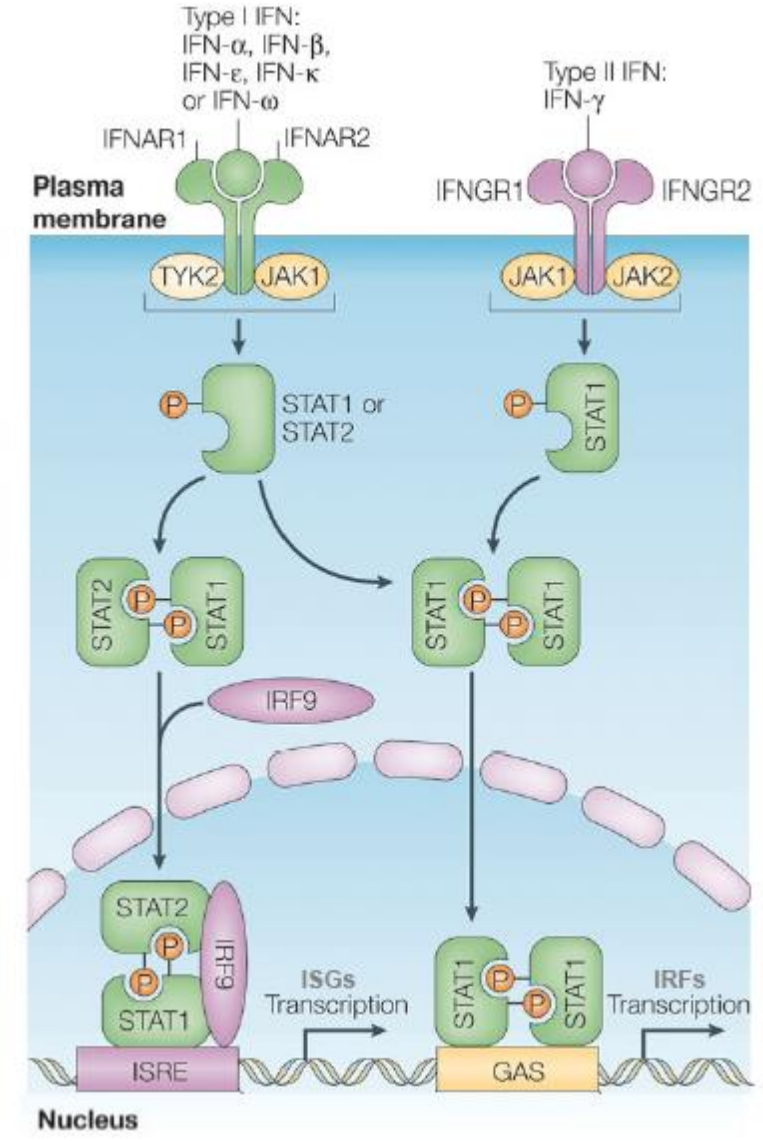

FIGURE 1: JAK/STAT activation as result of type I and typ II IFN activity.

Type I IFN interacts with IFNAR1 and IFNAR 2 on the target cells and activates JAK family members JAK1 and Tyk2. Activated JAKs phosphorylate STAT1 and STAT2, a process that leads to hetero dimerization of STATs. Dimerized STATs interact with IRF9 and form ISGF3 that binds to ISRE DNA sequence in the nucleus and activates expression of type I IFN sensitive genes (ISGs). Type II IFN interacts with IFNGR1 and IFNGR 2 subunits and activates JAK1 and JAK2 that phosphorylate STAT1. Phosphorylated STAT1 homo-dimerize and translocate to the nucleus and binds to GAS DNA sequence and activate expression of IFNγ sensitive genes.

The figure is modified from (Platanias, 2005)

JAK / STAT signaling is negatively regulated mainly by 3 classes of molecules; a) protein tyrosine phosphatases (PTPs) which inhibit the signaling by dephosphorylating the receptor and /

or activated JAKs and STATs, b) protein inhibitors of activated STATs (PIAS) which in some cases bind to dimerized STATs and inhibits their binding to DNA and c) suppressors of cytokine signaling (SOCS) that are expressed as result of JAK / STAT activation and bind to the JAKs and / or the IFN receptors in order to inhibit the signaling (Rawlings et al., 2004).

The Janus Kinases

JAKs are a family of large protein tyrosine kinases with sizes ranging from 120 to 140 kDa. In mammals four members of this family are identified; JAK1, JAK2, JAK3 and Tyk2 (Firmbach-Kraft et al., 1990, Wilks, 1989, Wilks, 1991, Harpur et al., 1992, Krolewski et al., 1990, Kawamura et al., 1994). Tyk2 was the first member of this family to be identified as an important molecule in formation of interferon stimulated gene factor 3 (ISGF3) as result of type I IFN signaling (Velazquez et al., 1992).

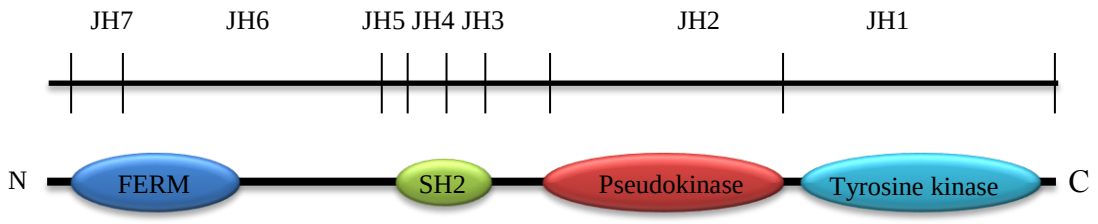

Figure 2. Domain like structure of JAKs

JAKs have a characteristic structure that is conserved between mammal, avian, teleost and insect species and it is divided into 7 JAK homology domains (JH) named from the carboxyl end (Figure 2). JH1 on the proteins C-terminal end is a tyrosine kinase domain (Wilks et al., 1991); it has a similar structure to other protein tyrosine kinases and includes an ATP binding site and a catalytic site. Specific mutation at the ATP binding site inactivates kinase function, probably by inhibiting ATP binding (Gauzzi et al., 1996). On the tyrosine kinase domain there are two tyrosine residues that are phosphorylated when the kinase is activated. The pseudo kinase domain

is located N-terminally to JH1, JH2 (Wilks et al., 1991) and shows all the hallmarks of protein tyrosine kinases but lacks kinase activity and it has been shown to act as a regulatory domain on tyrosine kinase. The kinase and the pseudo kinase domains seem to be evolutionary related (Wilks et al., 1991) . JH3 and JH4 constitute the Src homology 2 (SH2) like domain. And finally the JH6 – 7 domain which is named FERM (four point, ezrin, radixin, moesin) (Girault et al., 1999). The FERM domain seems to interact with transmembrane proteins like cytokine receptors (Richter et al., 1998, Zhao et al., 1995, Frank et al., 1995, Velazquez et al., 1995) and in the case of Tyk2, this interaction is necessary to sustain IFNRA level (Gauzzi et al., 1997) FERM also interacts with and positively regulates tyrosine kinase activity of JAKs. Inside the cell, JAKs are usually found in the cytosol, mostly associated with their receptors in the endosomes and at the plasma membrane (Yeh and Pellegrini, 1999). While JAK1, JAK2 and Tyk2 are constitutively expressed, JAK 3 is expressed only in hematopoietic cells and its expression is highly regulated through cell development and activation (Yeh and Pellegrini, 1999).

The Signal Transducers and Activators of Transcription

STATs are latent cytoplasmic transcription factors that are translocated to the nucleus upon activation and are found from mammals to insects and nematodes (Liongue and Ward, 2013). In mammals seven different STATs have been identified and named STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6 (Leonard and O'Shea, 1998). STAT molecules have highly conserved domains and characteristic structure comprised of; N-terminal domain, coil-coiled domain, DNA-binding domain (DBD), SH2 domain and transcription activation domain (TAD) (Figure 3).

Figure 3: Domain like structure of STATs

The N-terminal domain seems to be responsible for homo-dimerization of inactive STATs (Mertens et al., 2006, Mao et al., 2005) as well as their nuclear import, deactivation (Strehlow

and Schindler, 1998, Mertens et al., 2006) and their cooperative DNA-binding to tandem IFN-γ activation sequence (GAS) (Xu et al., 1996, Vinkemeier et al., 1996). The coiled – coil domain interacts with SH2 domain and facilitates its interaction with the receptor (Zhang et al., 2000). STAT coiled – coil is shown to interact with other proteins as well and in the case of STAT1 and STAT2 it is shown to bind other proteins like IRF9 (Horvath et al., 1996) and other regulatory proteins. The DBD binds to specific DNA sequences (Horvath et al., 1995, Ehret et al., 2001). Linker domain (LK) plays an important role for nuclear trans-location (Devaux et al., 2013) and in STAT1 it is essential for transcriptional activity of IFNγ (Yang et al., 1999, Yang et al., 2002) and in receptor recruitment and dimerization.The SH2 domain, is a sequence-specific phosphotyrosine-binding structure found in many molecules like JAKs and STATs. This domain is responsible for differential interaction of STATs with cytokine receptors in response to different stimuli (Hemmann et al., 1996) as well as homo and hetero dimeriziation of activated STATs (Gupta et al., 1996, Shuai et al., 1994). And finally the transactivation domain (TAD) is located at the C-terminus of the molecule (Muller et al., 1993, Moriggl et al., 1996, Caldenhoven et al., 1996, Mui et al., 1996). While tyrosine phosphorylation is obligatory for STAT activation, STATs are also phosphorylated on a serine residue located in the TAD domain (Ser^{727} in human STAT1), which has to be phosphorylated in order for STAT to exert full activity (Beadling et al., 1996). Both in STAT1 and STAT2 their TAD domains interact with p300 and CBP (CREB binding protein), which both are transcriptional co-activators (Bhattacharya et al., 1996, Zhang et al., 1996).

Interferon Regulatory Factor 9

Interferon Regulatory Factors (IRFs) are transcription factors involved in antiviral defense and immune response, as well as cell growth regulation and apoptosis. In human, nine members of this family are identified (IRF1 – 9) (Paun and Pitha, 2007, Nehyba et al., 2002).

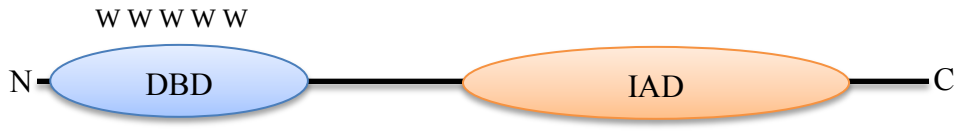

Figure 4: IRF9 structural domains

All IRFs have a highly conserved DBD in their N-terminal, with a helix – turn – helix formation that interacts with DNA through three of its five highly conserved tryptophan (W) residues (Escalante et al., 1998) (Figure 4). The C-terminus is more diverse and possesses regulatory functions (Barnes et al., 2002, Lin et al., 1999, Lin et al., 2000). Several members of the IRF family are involved in IFN signaling in various ways, IRF3, IRF7 and IRF5 are involved in expression of type I IFN genes (Juang et al., 1999, Wathelet et al., 1998), while IRF9 is the only member of IRF family that is directly involved in the JAK / STAT signaling pathway. In response to type I IFN stimulation STAT1, STAT2 and IRF9 interact and form ISGF3 that binds to a specific DNA sequence called interferon-stimulated response element (ISRE) (Fu et al., 1990, Levy et al., 1989). IRF9 in both mouse and human is shown to spontaneously accumulate in the nucleus because of a nuclear localization signal (NLS) present within or close to its DBD, but it is also detected in the cytoplasm of the cells (Lau et al., 2000).

Regulation of JAK / STAT

In mammals, the JAK/STAT pathway is the principal signaling mechanism for a wide array of cytokines and growth factors. Naturally, reduced activity, constitutive activity and / or failure in regulation of JAK / STAT signaling may lead to abnormality and disease; this makes its regulation essential. There are several molecules and processes involved in down-regulation of JAK/STAT signaling activity. Both JAKs and STATs are activated when they are phosphorylated on specific tyrosine residues (and serine in some cases) as result of external stimuli (i.e. IFN-stimulation), and one efficient strategy to down-regulate their activity is de-phosphorylation of these residues. Some of the major phosphatases involved in this process are; SH2-containing Protein Tyrosine Phosphatases 1 and 2 (SHP1 and SHP2), CD45, Protein Tyrosine Phosphatase 1B (PTP1B) and T-cell PTP (TCPTP).

SHP1 is expressed mainly in hematopoietic cells and is involved in de-phosphorylation of JAK1 (David et al., 1995) and JAK2 (Klingmuller et al., 1995, Jiao et al., 1996). CD45, a receptor PTP,

is a general inhibitor of JAKs and can directly bind to all JAKs and dephosphorylate them (Irie-Sasaki et al., 2001). PTP1B de-phosphorylates JAK2 and TYK2 (Myers et al., 2001) and TCPTP can de-phosphorylate JAK1 and JAK3 (Simoncic et al., 2002). SHP2 interacts with, and de-phosphorylates STAT5a (Chen et al., 2003, Chughtai et al., 2002) and seems to be involved in de-phosphorylation of STAT1. TC45 (the nuclear isoform of TCPTP) and SHP2 de-phosphorylate STAT1 in the nucleus (ten Hoeve et al., 2002, Wu et al., 2002), a necessary step for STAT1 nuclear export.

Another group of JAK / STAT inhibitors are Protein Inhibitors of STAT signaling (PIAS). Members of this group of proteins from drosophila to mammalian species are involved in negative regulation of STAT molecules. In mammals this group includes 4 members, PIAS1, PIAS3, PIASX and PIASY (Liu et al., 1998, Chung et al., 1997) . PIAS1 and 3 inhibit STAT1 and 3 respectively (Chung et al., 1997, Liu et al., 1998), by inhibiting their DNA-binding activity. PIASX inhibits STAT4 and PIASY interacts with STAT5 and regulates STAT5 mediated gene expression. PIASX and PIASY affect STAT4 and STAT1 activity without interfering with their DNA-binding activity probably by acting as co-repressors (Arora et al., 2003, Liu et al., 2001).

A third major group of proteins, and probably most studied group, involved in regulation of JAK / STAT signaling is a number structurally related proteins called Suppressors of Cytokine Signaling (SOCS). SOCS molecules are constitutively expressed at low rates, but their expression is up-regulated shortly after cytokine stimulation and they are degraded after a short time (Starr et al., 1997, Tollet-Egnell et al., 1999, Adams et al., 1998)**.** SOCS proteins inhibit type I and type II cytokines using different mechanisms; at the receptor level by competing with the STATs (JAK substrates), in binding to the intracellular domains of the activated receptors and on the JAK level, by interfering with their kinase activity. They are also involved in ubiquitination and proteosomal degradation of JAK and STAT molecules. The SOCS family includes 8 members; SOCS 1 – 7 and CIS or CISH (Cytokine-inducible SH2- containing protein) (Hilton, 1999, Hilton et al., 1998). They have similar structure which is comprised of a central SH2 domains, which like the SH2 domains in STATs has high affinity for phospho-tyrosine residues and defines specificity of SOCS, a variable N-terminal, and a 40 aa long sequence known as SOCS box in the C-terminal of proteins (Starr et al., 1997) which mediates ubiquitination and proteasomal degradation of their target molecules through interaction with the Elongin B and C, known

components of the ubiquitin E3 ligase complex. N-terminally to the SOCS1 and SOCS3-SH2 domain which interacts with tyrosine phosphorylated JAKs and inhibits their function (Endo et al., 1997, Starr et al., 1997). SOCS1 and SOCS3 have an additional domain called Kinase Inhibitor Region (KIR), a 12 aa long region which interacts with the kinase domain of JAKs and strongly inhibits their kinase activity (Yasukawa et al., 1999). SOCS1 and SOCS3 both inhibit JAK activity through their SH2 domain (Yasukawa et al., 1999), but while SOCS1-SH2 interacts directly with phospho-tyrosine residues of Tyk2 kinase domain to inhibit type I IFN signaling (Piganis et al., 2011) , SOCS3 – SH2 interacts with the phosphorylated tyrosine residues on the cytokine receptor and its KIR domain interacts with the substrate binding grove of JAK2 and blocks substrate association (Kershaw et al., 2013).

In addition to cytokine stimulation, SOCS are also expressed as a direct result of TLR stimulation by viral and bacterial factors like CpG-DNA (Dalpke et al., 2001) and LPS (Nakagawa et al., 2002). The effect that SOCS exert on cytokine responses in different ways has been used in immune evasion strategies of different pathogens (Zimmermann et al., 2006). Different pathogenic microorganisms seem to activate expression of SOCS by secreting factors such as lipo-proteins (Pai et al., 2003).

JAK / STAT in fish

Since discovery of IFNs in human in 1957 (Isaacs and Lindenmann, 1957) many different members of these family of cytokines have been identified, cloned and characterized both in mammalian species and other species of vertebrates like birds (Sekellick et al., 1994), amphibian (Grayfer et al., 2014) and fish (references cited below). IFN-like activity in fish was reported several decades ago (de Kinkelin and Dorson, 1973), but IFN from fish was cloned and characterized for the first time in 2003 in zebrafish *(Danio rerio)* (Altmann et al., 2003), puffer fish *(Tetraodontidae)* (Lutfalla et al., 2003), and Atlantic salmon *(Salmo salar)* (Robertsen et al., 2003) by 3 independent groups. Since then, IFN genes have been identified in several other teleost species like channel catfish (*Ictalurus punctatus*) (Long et al., 2004), common carp *(Cyprinus carpio L)* (Kitao et al., 2009), rainbow trout *(Oncorhynchus mykiss)* (Chang et al.,

2009), Rohu *(Labeo rohita)* (Parhi et al., 2014), sea bass *(Dicentrarchus labrax L.)* (Casani et al., 2009), Fugu *(Takifugu rubripes)*, goldfish *(Carassius auratus L)* (Grayfer and Belosevic, 2009) and Grass carp *(Ctenopharyngodon idella)* (Li et al., 2012).

Like mammalian type I IFN, fish IFNs have been shown to possess antiviral activity (Altmann et al., 2003, Robertsen et al., 2003) and their expression is upregulated upon viral infections (Collet et al., 2007) as well as stimulation with known inducers of mammalian IFNs like poly I : C (a synthetic mimic of ds RNA) and CpG DNA (Altmann et al., 2003, Robertsen et al., 2003, Pedersen et al., 2006, Chen et al., 2010). Several studies have established antiviral activity of recombinant fish IFNs in different species of teleost (Sun et al., 2011, Chaves-Pozo et al., 2010, Chen et al., 2014). Furthermore treatment of fish cells with IFNs are shown to induce of a wide range of IFN stimulated genes (ISG), including Mx, viperin, ISG15, PKR, and for some of these an enhanced antiviral state in fish cells are found (Chen et al., 2014, Sun et al., 2011, Svingerud et al., 2012).

While members of JAK / STAT signaling pathway have been extensively studied in different mammalian species, information about JAK / STAT signaling from fish is still scarce, even though molecules involved in this pathway are identified and characterized in several fish species. Studies of JAK molecules in round-spotted pufferfish (Leu et al., 1998, Leu et al., 2000) and the mandarin fish (*Siniperca chuatsi)* (Guo et al., 2009) have shown that JAKs in fish harbor the same structural composition and are of the same size as their mammalian counterparts. And studies in several fish showed that their expression is upregulated as a result of stimulation with IFNs and other IFN-signaling activators like CpG and poly I:C (Guo et al., 2009, Leu et al., 1998, Leu et al., 2000).

STAT1 and STAT2 have also been studied in different piscine species including Olive flounder *(Paralichthys olivaceus)* (Park et al., 2008) , pufferfish and in zebrafish (Stein et al., 2007), the mandarin fish *(Siniperca chuatsi)* (Guo et al., 2009), Atlantic salmon (Collet et al., 2008, Collet et al., 2009, Skjesol et al., 2010), crucian carp *(Carassius auratus L.)* (Zhang and Gui, 2004, Yu et al., 2010), turbot *(Scophthalmus maximus)* (Wang et al., 2013) and it has been shown that their expression is upregulated as result of CpG, poly I : C and IFN stimulation. The fact that Atlantic salmon STAT1 is the target of immune evasion for some viruses like IPNV and ISAV (Collet et al., 2008) and turbot STAT2 expression is upregulated in response to virus infection (Wang et al.,

2013), provide further evidence of involvement of teleost JAK/STAT in the organisms immune response to viral infection.

IRF9 from a few fish species have been identified (Shi et al., 2013, Shi et al., 2012). IRF9 homologue from crucian carp was shown to translocate spontaneously to the nucleus, while it was still detectable to some degree in the cytoplasm (Shi et al., 2012). IRF9 has both ISRE and GAS elements in its promoter region but in zebrafish its expression was primarily upregulated as a result of IFNγ stimulation. Transfection of carp epithelioma papulosum cyprini (EPC) cells with IRF3 and IRF7 as well as combination of IRF9 and STAT2, lead to activity of IRF9 promoter in luciferase assay (Shi et al., 2013).

Studies of SOCS in fish

In addition to ortologs to all SOCS found in mammals (SOCS1 – 7 and CISH) also several novel members of this family (SOCS-3b, SOCS-5b, SOCS-8 and SOCS-9) are found in teleost fish (Jin et al., 2008). Like SOCS from mammalian species, SOCS molecules from pufferfish, zebrafish, fugu,, japanese rice fish *(Oryzias latipes)* (Jin et al., 2007a, Jin et al., 2007b) and common carp (Xiao et al., 2010) possess similar domain like structures and have been expressed in all of the examined tissues.

The induction of fish SOCS gene expression is SOCS member-, cell type and cytokine dependent. Furthermore, like their mammalian counterparts, the fish SOCS are also induced by PAMPS and bacterial, viral and parasitic infections (Wang et al., 2011). For example in pufferfish, zebrafish, fugu, three-spined stickleback *(Gasterosteus aculeatus)* and the Japanese rice fish expression of SOCS 1 – 5, SOCS9 as well as CISH was upregulated as a result of LPS stimulation (Jin et al., 2007a). SOCS1 from both zebrafish and pufferfish were induced by IFN stimulation and its overexpression did inhibit expression of ISGs like Mx and Viperin (Nie et al., 2014). In fact SOCS1 is one of the main regulators of type I and II IFN signaling in mammals (Qing et al., 2005) as well as fish (Nie et al., 2014) and this also have been documented by our group (Skjesol et al., 2014). In rainbow trout stimulation with IFNγ, poly I:C and LPS lead to upregulation of SOCS1 – 3, but not of SOCS6, 7 and 9 (Wang et al., 2010, Wang and Secombes,

2008). Expression of Carp SOCS3 has been shown to be upregulated as result of virus infection (Xiao et al., 2010). Turbot SOCS3 homologue (named SmSOCS3) expression was upregulated as a result of bacterial infection as well as TNF stimulation and SmSOCS3 overexpression in HK macrophages inhibited the transcription of TNF-α as well as IL-1β and CC-chemokine (Zhang et al., 2011). These studies show that like their mammalian counterparts, SOCS molecules in bony fish are involved in regulation of inflammatory responses.

Aims of study

One of the main defense mechanisms activated in vertebrates in response to viral infection is the IFN activated JAK/STAT signaling pathway. On the other hand, in the course of their evolution viruses have developed strategies to counter this mechanism in order to infect the host organism. These strategies usually involve virus proteins disturbing different immune responses by attacking specific molecules involved in the immune response. A better understanding of molecules that are targeted and the processes that are inhibited by viruses is a necessity in order to design efficient vaccines against those viruses.

The aim of this study was to enhance understanding of JAK/STAT signaling in Atlantic salmon. To do so, major molecules involved in this pathway were cloned and/or characterized. The molecules cloned and characterized are:

- Tyk2
- STAT1a (characterization)
- STAT2 a/b
- IRF9
- SOCS1
- SOCS2 a/b
- CISH

Summary of papers

Paper I

Mehrdad Sobhkhez, Tom Hansen, Dimitar B. Iliev, Astrid Skjesol, Jorunn B. Jørgensen

The Atlantic salmon protein tyrosine kinase Tyk2: molecular cloning, modulation of expression and function. Dev Comp Immunol. 41(4): 553 – 63.

Tyk2, a member of the Janus Kinase (JAK) family of protein tyrosine kinases, is required for interferon-α/β binding and signaling in higher vertebrates. Currently, little is known about the role of the different JAKs in signaling responses to interferon (IFN) in lower vertebrates including fish. In this paper we report the identification and characterization of Atlantic salmon (Salmo salar) Tyk2. Four cDNA sequences, two containing an open reading frame encoding full-length Tyk protein and two with an up-stream in frame stop codon, were identified. The deduced amino acid sequences of the salmon full-length Tyk2 proteins showed highest identity with Tyk2 from other species and their transcripts were ubiquitously expressed. Like in mammals the presented data suggests that salmon Tyk2 is auto-phosporylated when ectopically expressed in cells. In our experiments, full-length salmon Tyk2 overexpressed in CHSE-cells phosphorylated itself, while both a kinase-deficient mutant and the truncated Tyk2 (Tyk-short) were inactive. Interestingly, the overexpression of full length Tyk2 was shown to up-regulate the transcript levels of the IFN induced gene Mx, thus indicating the involvement of salmon Tyk2 in the salmon IFN I pathway.

Paper II

Sobhkhez, M., Skjesol, A., Thomassen E., Greiner–Tollersrud L., Iliev, D.B., Sun, B., Robertsen B. & J. B. Jørgensen (2014)

Structural and functional characterization of salmon STAT1, STAT2 and IRF9 homologs sheds light on IFN signaling in teleosts. Manuscript.

Mammalian IRF9 and STAT2, together with STAT1, form the IFN-stimulated gene factor 3 transcription factor (ISGF3) complex, which is critical for type I IFN induced signaling, while IFN□ stimulation is mediated by homodimeric STAT1 protein. Teleost fish is known to possess

most JAK and STAT family members, however, description of their functional activity in lower vertebrates is still scarce. In the present study we have identified two different STAT2 homologs and one IRF9 homolog from Atlantic salmon (*Salmo salar* L). Both proteins have domain-like structures with functional motifs that are similar to higher vertebrates suggesting that they are orthologs to mammalian STAT2 and IRF9. The two identified salmon STAT2, named STAT2a and STAT2b, showed high sequence identity but were divergent in their transactivation domain (TAD). Like STAT1, ectopically expressed STAT2a and b were shown to be tyrosine phosphorylated by both type I IFNs and, interestingly also by IFN□. Microscopy analyses demonstrated that both STAT2a and STAT2b co-localized with STAT1a in the cytoplasm of unstimulated cells, while IFNa1 and IFN□ stimulation seemed to favor their nuclear localization. Overexpression of STAT2a or STAT2b together with STAT1a activated a GAS-containing reporter gene construct in IFNγ stimulated cells. Of notice, the highest induction of GAS promoter activation was found in IFNγ stimulated cells transfected with IRF9 alone. Taken together these data suggest that salmon STAT2 and IRF9 may have a role in IFN□ induced signaling and promote the expression of GAS-driven genes in bony fish.

Paper III

Astrid Skjesol, Theresa Liebe, Dimitar B. Iliev, Ernst I Thomassen, Linn G. Tollersrud, Mehrdad Sobhkhez, Lisbeth Lindenskov Joensen, Chris J. Secombes and Jorunn B. Jørgensen

Functional conservation of suppressors of cytokine signaling proteins between teleosts and mammals: Atlantic salmon SOCS1 binds to JAK/STAT family members and suppresses type I and II IFN signaling. Dev Comp Immunol 45(1): 177 – 89.

Suppressor of cytokine signaling (SOCS) proteins are crucially involved in the control of inflammatory responses through their impact on various signaling pathways including the JAK/STAT pathway. Although all SOCS protein family members are identified in teleost fish, their functional properties in non-mammalian vertebrates have not been extensively studied. To gain further insight into SOCS functions in bony fish, we have identified and characterized the Atlantic salmon (Salmo salar) SOCS1, SOCS2 and CISH genes. These genes exhibited sequence conservation with their mammalian counterparts and they were ubiquitously expressed. SOCS1 in mammalian species has been recognized as a key negative regulator of interferon (IFN)

signaling and recent data for the two model fish Tetraodon (Tetraodon nigroviridis) and zebrafish (Danio rerio) suggest that these functions are conserved from teleost to mammals. In agreement with this we here demonstrate a strong negative regulatory activity of salmon SOCS1 on type I and type II IFN signaling, while SOCS2a and b and CISH only moderately affected IFN responses. SOCS1 also inhibited IFNγ-induced nuclear localization of STAT1 and a direct interaction between SOCS1 and STAT1 and between SOCS1 and the Tyk2 kinase was found. Using SOCS1 mutants lacking either the KIR domain or the ESS, SH2 and SOCS box domains showed that all domains affected the ability of SOCS1 to inhibit IFN-mediated signaling. These results are the first to demonstrate that SOCS1 is a potent inhibitor of IFN-mediated JAK-STAT signaling in teleost fish.

Discussion of the main findings.

All our findings have been thoroughly discussed in attached papers (papers: I, II and III). The following section will focus on parts of the finding and will complement the discussions in the published papers and manuscripts.

Tyk2

Tyk2 and JAK1 associate with intracellular domain of type I IFN receptor while JAK1 and JAK2 with that of type II IFN. Since Tyk2 is the kinase specifically involved with type I IFN signaling and many viruses inhibit JAK / STAT signaling as part of their strategy to evade antiviral immunity, we decided to clone and characterize Tyk2 from Atlantic salmon. This was motivated by earlier data published by our group where we could demonstrate that IPNV is able to antagonize type I IFN-signaling in different salmonid cell-lines (Skjesol et al., 2009) and by unpublished data suggesting that the virus target proteins at or upstream of STAT1 phosphorylation (A. Skjesol and T. Hansen, unpublished data).

Our efforts lead to identification of four different isoforms of salmon Tyk2, ssTyk2-1 to 4, two of which (1 and 4) encode full length molecules with a conserved structure among vertebrate JAKs containing a C -terminal: kinase, followed by a pseudokinase, and then a SH2-homology and a FERM domain. Tyk2-2 and Tyk2-3 were shorter molecules and their sequences included only the FERM domain and part of the SH2 domain. Moreover, phylogenetic analysis indicated that salmon Tyk2s are true orthologs of the mammalian Tyk2. Reports from other organisms have shown that different factors involved in JAK/STAT signaling are expressed in all tissues that were studied (Yang et al., 1993, Stark and Darnell, 2012, Shuai and Liu, 2003). This was also the case here; Tyk2 was expressed in all organs from healthy salmon with some variations in the expression level. In addition, studies of the primary leucocytes from head kidney and spleen as well as CHSE-214 and TO cell lines showed evidence of upregulated Tyk2 mRNA upon both type I and II IFN stimulation.

When JAKs are brought in the proximity of each other they are activated through phosphorylation of tyrosine residues located in their kinase domain. Our studies showed that overexpressed Tyk2 is spontaneously tyrosine phosphorylated, and this phosphorylation occurs on the tyrosine residues located in the kinase domain. This became evident when we expressed and studied different domains of Tyk2 separately. We didn't observe any phosphorylation of tyrosine residues in the point mutated Tyk2 (Tyk2 K917R) even though tyrosine residues which are phosphorylation targets were intact. This led us to conclude that the phosphorylation observed was due to auto-activity and not caused by the activity of another kinase.

Tyk2 is described as an important component for an efficient induction of ISGs. Our studies showed that auto-phosphorylated Tyk2 was able to increase Mx-promoter activity in CHSE cells using a luciferase reporter construct, something that neither Tyk2-short (Tyk2-3) nor the Tyk2 K917R mutant were able to do. Furthermore, overexpressing Tyk2 in CHSE cells lead to upregulated Mx transcript levels, something that was not detected in control cells (cells only transfected with GFP-empty vector or Tyk2 K917R). These data provided strong indication of Tyk2 being activated when it is in proximity of other Tyk2 molecules; this activity is due to tyrosine phosphorylation of the kinase domain and Tyk2 activation leads to expression of ISGs like Mx.

STAT1 & 2

Activation of JAKs as result of type I IFN stimulation leads to phosphorylation and hetero dimerization of STAT1 and 2 and their association with IRF9. These three factors together constitute Interferon Stimulated Gene Factor 3 (ISGF3). Formation and translocation ISGF3 to the nucleus and its binding to specific DNA sequences lead to expression of many ISGs.

Earlier studies from our research group had already stated that salmon STAT1a was tyrosine phosphorylated upon IFNa1 and IFNγ stimulation(Skjesol et al., 2010). Our attempts to further characterize the molecule lead to identification of the tyrosine target for phosphorylation as result of IFN stimulation. We showed that this tyrosine (tyrosine 695) is located in the SH2 domain. Interestingly, no phosphorylated tyrosine residues were detected when the SH2 domain itself was

ectopically expressed, something that indicated requirement of the whole STAT1a sequence for tyrosine phosphorylation. Further studies showed that STAT1a is tyrosine phosphorylated when cells are stimulated with IFNa1, IFNc and IFNγ and that it translocated to the nucleus when cells transiently transfected with STAT1a were stimulated with IFNa1 and IFNγ.

Our search for salmon STAT2 resulted in identification and characterization of two isoforms - STAT2a and STAT2b. In addition, there is a previously identified STAT2 homolog (Collet et al., 2009) which we named STAT2c. As expected, all STAT2 isoforms displayed conserved structural domains, like other STAT molecules. All three isoforms had high aa identity with each other and their aa sequences were identical in N-terminus, STAT2a and b were also identical in coiled – coil domain and different from STAT2c, while STAT2a and c were identical in TAD domain which was different from STAT2b TAD. Since it has been reported that the coiled-coil domain is involved in protein interactions and that the TAD domain does interact with transcription co-activators, it seems that these different sequences are involved in recruiting different molecules and could be involved in different activities of STAT2 in salmon.

While as previously shown STAT1a did translocate to the nucleus as result of IFN stimulation (Skjesol et al., 2010), when STAT2a/b were expressed alone they did not translocate. However, they did translocate to the nucleus in response to IFN stimulation when they were co-expressed with STAT1a. This was an interesting observation that needs to be explained. One possible explanation would lie on the availability of endogenous STAT1. If STAT1a is available in lower concentration then it could mean that there was need for STAT1a over-expression in order for STAT2 to translocate. We know that STAT1a protein expression is upregulated when cells are stimulated with IFN. But in general it seems that STAT2 needs hetero dimerization in order to translocate to the nucleus, this leaves a lingering question that needs to be answered; does salmon STAT2 have a functioning NLS sequence? This can only be answered by additional experiments.

IRF9

Last major molecule involved in ISG3 formation is IRF9. Study of the salmon IRF9 sequence revealed that this molecule had the classic IRF molecule structure, with a DBD which included

five conserved tryptophan (W) residues in exactly the same position as in mammalian IRF9. At the end of the DBD there is one segment of aa identified by online NLS finding software (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) as a possible NLS. Also, an IAD domain is located at the C-terminus of the molecule. This domain showed high divergence compared to IRF9 sequences from other species.

Since direct interaction between Tyk2 and STAT molecules as well as interaction between STAT1, STAT2 and IRF9 is essential in formation of ISGF3 and ISG expression in mammals, we wanted to find out whether these interactions exist in salmon. Our co-IP and confocal microscopy data showed that these molecules did indeed interact and co-localized with each other.

We found out that overexpressed salmon IRF9 spontaneously accumulated in the nucleus and in reporter gene assay it activated a GAS element when stimulated with IFNγ. In addition, STAT2a and b alone also activated the GAS element upon IFNγ stimulation, while, STAT1a did not do that. This could be due to the experimental limitation. For these experiments we used CHSE-214 cell-lines and the transient transfections showed varying efficiency which might have influenced the results. To be able to discuss the role that IRF9 plays in GAS activity in salmon compared to higher vertebrates, one has to perform similar experiments in a mammalian setup , only then it's possible to discuss these finding in comparison with the role that IRF9 plays in higher vertebrates and to reveal a possible difference in the role that IRF9 plays in salmon compared to human or rat.

SOCS

Activated JAK STAT signaling is necessary for anti-viral activity of the cells. Lack of regulation or spontaneous activity of the signaling can cause many unwanted affects like auto-immunity. That's why JAK STAT activity is tightly regulated. SOCS molecules are one of the most important regulatory molecules.

To shed light on the regulation of Atlantic salmon JAK/STAT signaling three members of this family were cloned: SOCS1, SOCS2 (two isoforms: a and b) and CISH. The structural

arrangements for all three SOSC-family members identified here were similar to the mammalian SOCS proteins as discussed in paper III. We studied possible inhibitory effect of SOCS proteins on different IFN induced promoters using reporter gene assay. While our data showed moderate negative regulatory effect of SOCS2a, SOCS2b and CISH on both IFNa1 and IFNγ signaling, SOCS1 managed to completely abolish effect of both IFNa1 and IFNγ on the promoter activity. Like Tyk2, STAT1, STAT2 and IRF9, also SOCS molecules are expressed in all tissues of healthy salmon that were included in the study and their expression was regulated (up-regulated) by IFN stimulation as well as the IFN inducing immunostimulants CpG and poly I:C. Our results showed that while different domains of the molecules have variable impact on the regulation of JAK / STAT signaling, the full-length molecules are required for full function. SOCS1 like its mammalian counterpart did translocate to the nucleus and co-expression of SOCS1 with STAT1 inhibited nuclear translocation of STAT1a upon IFNγ stimulation.

Future prospects:

While we tried to expand this study as much as possible – given the time we had – to answer main questions related to Atlantic salmon Tyk2, STAT1, STAT2 and IRF9 and their role in IFN-signaling in bony fish, there are many questions that remain to be answered.

The fact that STAT2a, b, and c have different aa-sequences in essential domains (coiled-coil and TAD) speaks of their possibility to interact with different cellular regulators and co-activators. Identification of those molecules could provide us with information about possible novel roles JAK/STAT signaling may have in bony fish for example shed light on the possible role STAT2a and STAT2b play in activation of GAS element.

We identified putative NLS sequences in STAT1, STAT2a, STAT2b and IRF9 by aa-identity to already characterized NLS from mammalian molecules. It remains to be proven if these sequences do have the expected function in salmon or not. This is especially important for the STAT2 isoforms. The fact that STAT2 is only translocated to the nucleus when it is co-expressed with STAT1a or IRF9, questions the presence of a functional NLS sequence in salmon STAT2.

And finally the most interesting finding in this study is the stimulatory role that IRF9 may have for GAS element. To our knowledge earlier findings have only documented that IRF9 and unphosphorylated STAT2 are implicated in IFNγ signaling through the formation of $ISGF^{II}$ and ISRE activation (Morrow et al., 2011). Our findings indicate that they may have a stimulatory role on GAS element. This certainly needs further elucidation. One way to do so is to introduce point mutations on the tryptophan residues in DBD that are essential for IRF9 function. Elimination or reduction of GAS activity in cells transfected with mutant IRF9 upon stimulation with IFNγ could be done to elucidate weather IRF9 has a role in the activation of GAS element. Of course here it's important to consider the shortcomings of the methods used. While reporter gene assay is broadly used, it has its shortcomings. The result could be affected by the cell-type, transfection efficiency, endogenous molecules present and the fact that this method excludes the effects of distant enhancers that are present in the genome.

Main conclusion

- Tyk2, STAT2 (two isoforms), IRF9, SOCS1, SOCS2 (two isoforms) and CHISH from Atlantic salmon have been cloned.
- Overexpressed Tyk2 is auto-activated as result of tyrosine phosphorylation and this leads to expression of ISGs in CHSE-214 cells.
- STAT1a and STAT2 a/b are phosphorylated as result of type I and II IFN stimulation.
- Tyk2, STAT1a, STAT2a/b and IRF9 interact with each other.
- IRF9 spontaneously accumulates in the nucleus when overexpressed.
- STAT2a/b translocate to the nucleus when co-expressed with STAT1a as result of both type I and type II IFN stimulation
- STAT2a/b translocate to the nucleus when co-expressed with the IRF9.
- IRF9 seems to activate GAS element in CHSE-214 cells.
- Expressions of all these molecules are up-regulated as result of IFN stimulation, this shows that these molecules are ISGs themselves.

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Paper I

Paper II

Paper III