A Functional Study of the Major Histocompatibility Class I Antigen Presentation Pathway in Rainbow Trout *(Oncorhynchus mykiss)*

by

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I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Major Histocompatibility Complex (MHC) class I receptors are glycoproteins which play a critical role in anti-viral immunity by displaying foreign peptides to cytotoxic T cell lymphocytes. The loading of high affinity peptides into the MHC class I receptor in mammals is coordinated by a multiple proteins that are collectively referred to as the peptide loading complex (PLC). To date, the composition of the peptide loading complex in fish is unknown and therefore the characterization of the molecules which may exist in this putative complex was pursued.

This thesis includes the cloning and functional characterization of ERp57 and calnexin in rainbow trout which, in mammals, are known to interact with the MHC class I receptor either during its early biogenesis or later in the assembly of the PLC. Trout ERp57 and calnexin cDNA sequences are ubiquitously expressed in trout tissues and both the ERp57 and calnexin genes appear in at least two copies each in the trout genome. Interestingly, despite their high sequence identity with their mammalian homologues, some structural discrepancies were identified. ERp57 does not contain an endoplasmic reticulum (ER) retention signal or a nuclear localization signal, while one of the two isolated cDNA clones for calnexin does not contain an ER (endoplasmic reticulum) retention signal and lacks a conserved C-terminal serine phosphorylation site. These findings suggest that in trout, there may be unique versions of these proteins that have acquired different cellular functions. Through the production of polyclonal antibodies against trout ERp57, the conserved protein induction of ERp57 during ER stress was demonstrated concurrently with calnexin.

In addition, this study shows for the first time that ERp57 can be induced transcriptionally by phytohemagglutinin and synthetic double stranded RNA, which implies its possible regulatory role during viral infection and the activation of the immune response. Furthermore, the functional characterization of the MHC class I specific chaperone tapasin, a key element in the PLC of mammals was pursued. Tissue and cell line distribution revealed that tapasin is expressed in high levels in immune system organs and in the rainbow trout macrophage cell line RTS11, at a relative molecular weight of 48 kDa with an additional 20 kDa band detected by the tapasin antibody. Tapasin protein was significantly up regulated upon exposure to synthetic double stranded RNA and during infection with two fish viruses: chum salmon virus and viral hemorrhagic septicemia virus genotype IVa, whereas the expression of the 20 kDa band was not affected by these stimuli.

This study also examined the regulation of the MH class I heavy chain, $\beta 2$ microglobulin and their associated machinery upon exposure to viral hemorrhagic septicemia virus

genotype IVa at permissive and non-permissive temperatures. β2 microglobulin secretion into the cell media, a marker of MH class I receptor turnover, was detected in the conditioned media of RTS11 cells under normal conditions and was shown to be significantly enhanced during viral hemorrhagic septicemia virus genotype IVa infection. Furthermore, when RTS11 cells were maintained at cold temperatures, the secretion of β2 microglobulin was significantly reduced in both infected and non-infected cultures, while the cellular levels of β2 microglobulin remained unchanged. These results suggest that cold temperature can alter the expression of the MH class I molecule on the cell surface and therefore may be contributing to host susceptibility to viral hemorrhagic septicemia virus genotype IVa during the winter.

Lastly, Co-immunoprecipitation demonstrated the interaction of the lectin chaperones: calnexin and calreticulin with the glycosylated MH class I receptor supporting their conserved role during MH class I receptor folding in fish. Concurrently, tapasin's interaction with transporter associated with antigen processing (TAP) and with the glycosylated form of the MH class I was revealed for the first time in fish, which supports their role in antigen presentation as in mammals. This study demonstrated that ERp57 and tapasin form a conserved disulfide linked heterodimer of 110 kDa, however unlike mammals, an additional 75 kDa heterodimer was detected which suggests a possible novel interaction of ERp57 with a 20 kDa tapasin version alternately regulating antigen presentation in fish.

Overall, this study suggest that the interactions involved in antigen presentation in mammals are conserved in fish, however the presence of different protein versions of calnexin, ERp57 and tapasin might dictate a different mode of regulation for MH class I assembly in fish, as opposed to mammals. Elucidating these interactions during various viral infections in fish can help to uncover possible viral strategies to manipulate the host immune response and will provide information needed to assist in designing novel tools to prevent fish viral diseases.

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Dedication

This is dedicated to my parents Hadassa and Refael Barda which I enormously love.

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List of Abbreviations

aa	Amino acids
ABC	ATP binding cassette
APC	Antigen presenting cell
β2m	β2 microglobulin
cDNA	Complementary DNA
CSV	Chum salmon virus
CTL	Cytotoxic T lymphocyte
Cys	Cysteine
DIG	Digoxigenin
dNTP	deoxynucleotide triphosphate
dsRNA	Double stranded RNA
DRiP	Defective ribosomal products
EndoH	Endoglycosidase H
EPC	Epithelioma carp papulosum cell line
ER	Endoplamic reticulum
ERAAP	ER aminopeptidases
ERSE	ER response elements
FBS	Fetal bovine serum
GAS	Interferon γ activated sequence
Gls	glucosidase
HC	Heavy chain
HCMV	Human cytomegalovirus
HSV	Herpes simplex virus
IFN	Interferon
Ig	Immunoglobulin
IHNV	infectious hematopoietic virus
IP	immunoprecipitation
IRF	Interferon regulatory factor
ISAV	Infectious salmon anemia virus
ISG	Interferon stimulating gene

JAK	Janus activated kinase
kDa	KiloDalton
L-15	Leibovitzs media
LMP	Low molecular weight polypeptide
MH	Major histocompatibility
MHC	Major histocompatibility complex
MMTS	Methyl methanethiosulfonate
mRNA	Messenger RNA
NBD	Nucleotide binding domain
NLS	Nuclear localization signal
PBL	Peripheral blood leukocytes
PBR	Peptide binding region
P.I.	Post infection
RACE	Rapid Amplification of cDNA Ends
TAP	Transporter associated with antigen processing
TAPBP	Tapasin binding protein
Th	T-helper
TLR	Toll like receptor
TRX	Thioredoxin
TMD	Transmembrane domain
UGT	UDP-glucose transferase
PBL	Peripheral blood leukocytes
PCR	Polymerase chain reaction
PDI	protein disulphide isomerase
PHA	Phytohaemagglutinin
PLC	Peptide loading complex
Poly IC	Polyinosinic-polycytidylic acid
PRR	Pattern recognition receptor
Rig	Retinoic acid-inducible gene
RNA	Ribonucleic acid
RT–PCR	Reverse transcription-PCR
RTS11	Rainbow trout spleen macrophage-like cell line
RTG-2	Rainbow trout gonadal fibroblast-like cell line
SSC	Saline sodium citrate

SSH	Suppressive subtractive hybridization
STAT	Single transducer and activator of transcription
TGFβ	Transforming growth factor β
TRX	Thioredoxin
VHSV	Viral hemorrhagic septicemia virus
VSV	Vesicular stomatitis virus

Chapter 1

General Introduction

The overall goal of this thesis is to explore the mechanisms involved in major histocompatibility (MH) class I antigen presentation in fish. This process loads peptides into specialized grooves in the major histocompatibility receptors. Although sequence similarity, tissue distribution and polymorphism suggest that these molecules function similarly to those in mammals, little is known about the functional role of MHC receptors during disease in fish. Elucidating the mechanisms carrying out antigen presentation in fish can help to understand the fish immune system and can be used in aquaculture for vaccine design, selective breeding and disease protection.

1.1 The MHC class I complex

The human major histocompatibility complex (MHC) is a large genetic complex on chromosome 6 which spans over four million base pairs and includes over 220 identified genes [1]. The human MHC genes are clustered in distinct linked regions: class I, class II, and class III. Within the class I region reside the MHC class I genes and additional non-immune related genes, whereas the class II region encodes the MHC class II class II α and β genes along with with numerous genes associated with antigen processing. The class III region consists of genes encoding both non-immune and immune related genes such as complement proteins, inflammatory cytokines and heat shock proteins. Remarkably, the MHC gene cluster has been isolated in all jawed vertebrae including birds, frogs and sharks which appear to be the earliest group of vertebrae to acquire both the both the MHC class I and II genes [2]. The first evidence for the functional role of the MHC came from studies done

in the early 1930s by Peter Gorer which linked tumour graft rejection in mice with transplantation antigens which were later referred by George Snell as the histocompatibility (H) genes and were mapped in mice to the region named the H2 locus [3]. The human MHC genes were found a decade later by Jean Dausset and were referred to as human leukocyte antigen (HLA) genes. The "artificial role" of the MHC during transplant rejection led the way for their discovery, however the natural role of the MHC was revealed in the 1970s in studies conducted with inbred mice and guinea pigs which demonstrated that the immune response against foreign antigens is genetically controlled by the MHC genes [4, 5]. After a few decades of intensive research it has now been demonstarted that the MHC class I and class II play an important role during the immune response by presenting foreign peptides to T cells, however the source of the peptides and the intracellular route in which they are acquired is mostly unique to each class of MHC proteins.

1.1.1 The MHC proteins

MHC class I receptors are expressed on the cell surface of all nucleated cells and classically present peptide fragments of intracellular origin (e.g. viral peptides) to cytotoxic T lymphocytes (CTL) which are also referred as CD8+ cells [6]. In contrast to the wide distribution of MHC class I receptors, MHC class II receptors are expressed only on antigen presenting cells (APC) such as B lymphocytes, dendritic cells and macrophages and mainly display peptides derived from extracellular origin (e.g. bacterial peptides) to T helper lymphocytes (Th), also referred as CD4+ cells [7].

Both MHC class I and class II receptors are non-covalent heterodimers: The MHC class I protein is composed of a polymorphic heavy chain (HC) associated with a soluble light chain $\beta 2$ microglobulin ($\beta 2$m) encoded by a non-MHC linked gene. This association is necessary for the proper folding, delivery and surface stability of the MHC class I molecule with the bound peptide [8]. The MHC class II protein, on the other hand, comprises two chains the α and β both of which are encoded within the MHC class II region [9]. In contrast to $\beta 2$m, both the MHC class I heavy chain and the MHC class II chains contain a transmembrane domain and a short cytoplasmic tail (Figure 1.1.1). The extracellular region of the heavy chain is folded into three extracellular domains $\alpha 1$, $\alpha 2$ and $\alpha 3$, however, only the $\alpha 1$ and the $\alpha 2$ makes up the the peptide binding region (PBR) located in the distal part of the receptor, which carry the polymorphic signature of the MHC molecule [10]. In contrast, MHC class II chains α and β are each folded into two distinct domains

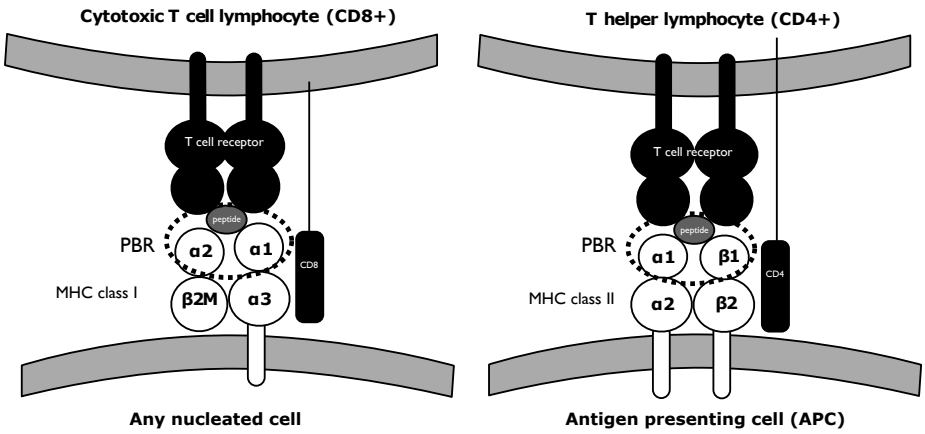

Figure 1.1.1: **The MHC class I and class II receptors function is to present antigenic peptides to T cell lymphocyte.**
The $\alpha 1$ and $\alpha 2$ domains of the MHC class I molecule comprise the peptide binding region (PBR) which binds mainly to intracellular peptides and presents them on the cell surface for cytotoxic T cell lymphocytes (CD8+), whereas the $\alpha 1$ and $\beta 1$ domains of the MHC class II molecule comprise the PBR which binds to extracellular peptides which are displayed on the cell surface for binding by helper T cell lymphocytes (CD4+). The PBR region is indicated by a dashed circle.

$\alpha 1$ $\alpha 2$ and $\beta 1$, $\beta 2$ respectively [9]. Both the $\alpha 2$ and $\beta 2$ are adjacent to the membrane, whereas the $\alpha 1$ and $\beta 1$ fold together to form the peptide-binding groove [3].

The crystal structure of the MHC proteins revealed three dimensional structure of the peptide binding region composed of two α helical regions forming its sides and eight β sheet strands constituting the platform [11, 12, 13]. The peptides that bind to the MHC class I groove are mostly 8-10 amino acids long and are anchored to the PBR at both C and N termini of the peptide. In contrast, the peptide binding to the MHC class II protein is not as constrained, allowing the binding of longer peptides of 13 to 20 amino acids long that can extend outside the ends of the PBR. Importantly, amino acid substitutions within the peptide binding region of both MHC class I and class II proteins are the main determinants of peptide specificity, allowing MHC alleles to bind a diverse pool of peptides and therefore facilitating better protection against a wide range of pathogens [3].

1.1.2 Antigen processing

The loading of the MHC class I receptor with a peptide takes place in the endoplasmic reticulum (ER) and requires a supply of peptides from the cytosol which need to be deliv-

ered into the lumen of the ER. Peptides are generated in the cytosol by the proteasome, a cylindrical 20S core structure composed of four heptameric rings. The external rings are composed of α subunits (α1-α7) and form the entry gates, whereas the inner rings contain the β subunits (β1-β7) that comprise the active sites: β1, β2 and β5. In response to induction by interferon, these catalytic subunits can be substituted to form a the immunoproteasome by the proteins LMP2 (low molecular mass polypeptide 2), LMP7 and MECL-1 [14]. The immunoproteasome alters the peptide repertoire by enhancing cleavage after hydrophobic or basic amino acids residues and preventing the cleavage after acidic amino acids. This type of cleavage results in peptides with a carboxy terminus that is more suitable for the delivery to and binding of MHC class I [14].

As opposed to the proteasomal cleavage of proteins, another important and rapid source of peptide supply is from defective ribosomal products (DRiPs) which are proteins derived from defective transcription or translation products that comprise between 30% to 70% of the cellular protein pool. These aberrant protein products are instantly sent for degradation by the proteasome and therefore provide a quick supply of antigenic peptides. The generation of DRiPs can be advantageous during viral infection allowing rapid presentation of foreign peptides to T lymphocytes which can then kill the infected cells shortly after viral proteins are produced in the cytosol [15]. Most of the peptides produced by the proteasome are either too long or too short (3-22 amino acid long) to accommodate the MHC class I binding groove. However, ER aminopeptidases (ERAAP) can further cleave the N terminal end of the peptides, allowing them to fit the peptide binding groove [16].

1.1.3 Antigen delivery

The peptide supply from the cytosol is transported into the lumen of the ER by a special ATP dependent heterodimer named the Transporter Associated with Antigen Processing (TAP) which in mammals is encoded by two genes in the MHC class II region closely linked to LMP2 and LMP7 genes. The TAP is a member of the ATP binding cassette (ABC) family of transporters which contains two N terminal transmembrane domains (TMD) forming the translocation pore and two cytoplasmic nucleotide binding domains (NBD). The peptides that bind TAP can vary in length between 8-40 residues, however peptides of 8-16 amino acids with hydrophobic and basic C terminal residues are preferred both for binding and transport. The delivery of peptides into the ER lumen from the cytosol is executed by cycles of ATP binding and hydrolysis, which is linked to conformational changes in the TMD domains [17]. Evidence for the important role of TAP during antigen

presentation came from studies using TAP deficient mice that displayed poor levels of MHC class I on the cell surface and failed to present endogenous peptides to cytotoxic T cell lymphocytes [18] indicating that peptide binding is a requirement for MHC class I expression on the cell surface.

1.2 MHC class I peptide loading complex

Once the peptides reach the ER they are destined to bind a wide variety of MHC alleles each with a different binding affinity, therefore a specialized pathway has evolved in the ER that ensures the proper folding of the MHC class I receptor with the correct high affinity peptide [19]. In humans, the newly synthesized class I heavy chain associates initially with ER lectin chaperone calnexin that spans the ER membrane. When class I heavy chain assembles with β2m, calnexin is displaced by its soluble homologue calreticulin which together with additional protein components such as the chaperone tapasin, the protein disulfide isomerase ERp57 and the heterodimer TAP, makes up the PLC which facilitates the optimal folding requirements of the MHC class I molecule and the loading of high affinity peptides. [6, 8] (Figure 1.2.1, Figure 1.2.2).

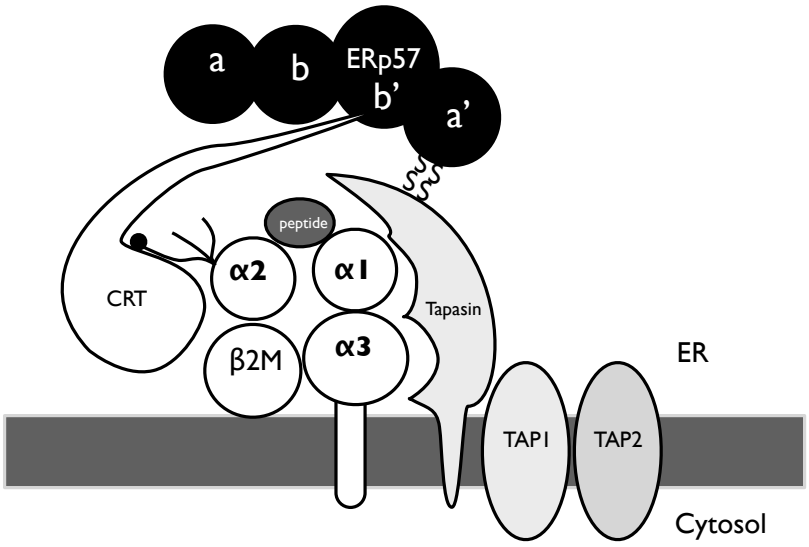

Figure 1.2.1: **The peptide-loading complex (PLC).**
In humans, the peptide loading ensures the proper folding of the MHC class I molecule with high affinity peptides before its export to the cell surface. The PLC consists of TAP, the MHC class I heterodimer, calreticulin, tapasin conjugated to ERp57 and the antigenic peptide.

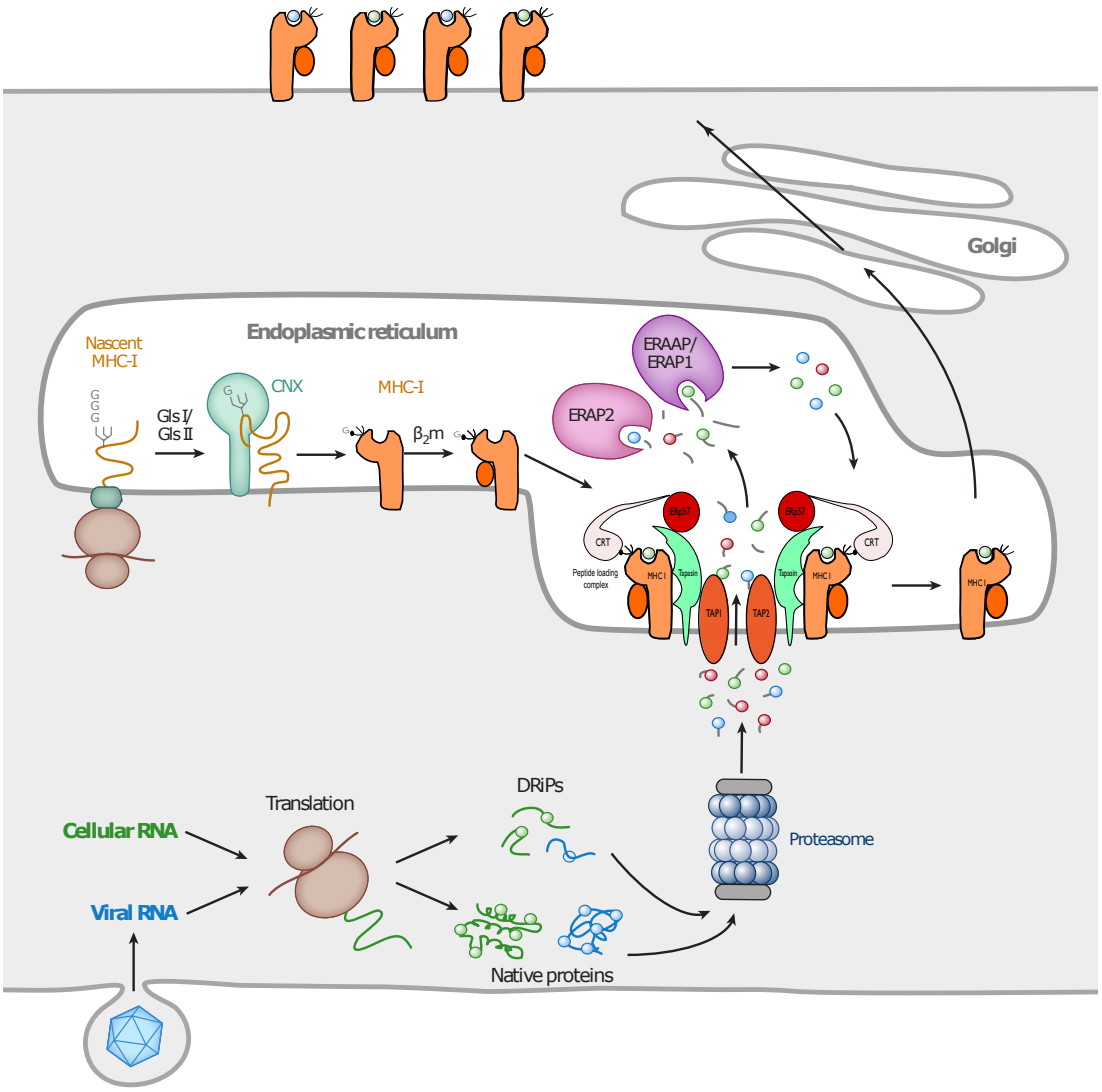

Figure 1.2.2: **The MHC class I assembly in the endoplamic reticulum.**

During early folding of the MH class I heavy chain, the lectin chaperone calnexin will bind to the glycosylated MHC class I heavy chain. Upon assembly with $\beta 2m$, calnexin will be released and substituted by calreticulin to bind the MHC class I-$\beta 2m$ and to ERp57 conjugated to tapasin to form the PLC. The peptides are derived from native protein or from DRiPs which are degraded by the proteasome and translocated into the ER lumen by TAP where they can undergo further trimming by ERAAP. If the cell is presenting endogenous foreign peptide, it will be destroyed by CD8+ cells. Adapted from [20].

1.2.1 Calnexin and calreticulin

Calnexin (IP90/P88) is a 90 kDa ER integral protein which binds to monoglucosylated N-linked glycans. It is an evolutionarily conserved protein that shares functional and structural domains with calreticulin, its soluble homologue [21]. Both calnexin and calreticulin share a conserved region referred as the P domain that contains two proline rich motifs repeated three times each in calreticulin and four times each in calnexin [22, 23]. The N terminal domain of calnexin and calreticulin contains the lectin binding site that mediates binding to a single glucose residue present on N-linked glycan intermediates, generated by the competing enzymatic action of glucosidase I/II (GlsI/GlsII) and UDP-glucose transferase 1 (UGT-1), which trims and restores the glucose respectively until the glycoprotein reaches optimal folding. Calnexin and calreticulin can also bind non-glycosylated proteins and possess polypeptide binding properties which can prevent aggregate formation of both glycosylated and non-glycosylated proteins [24, 25].

Apart from their established role as molecular chaperones, calnexin and calreticulin have also been shown to play a role in cellular calcium homeostasis by binding calcium in the ER [26]. Another interesting role of calreticulin was demonstrated recently which showed that calreticulin encoded by *Trypanosoma carassii* can evade the immune response by bind host complement C1q and inhibits classical complement pathway-mediated lysis [27].

One of the well investigated functions of calnexin, which also contributed to its initial identification, is its association with MHC class I receptor [28, 29]. In mice, calnexin has been shown to bind the heavy chain-β2m heterodimer in the ER, however in humans this interaction could not be identified and only association with the class I heavy chain has been observed [8, 6]. The functional significance of calnexin's interaction with MHC class I has been controversial. On one hand, it was demonstrated using a *Drosophila melanogaster* cell line that co-expression of calnexin can enhance the folding efficiency of mouse MHC class I heavy chain with β2m. In addition, by using ER glucosidase inhibitors, which prevent calnexin from interacting with its substrates, MHC class I surface expression was reduced. These finding supported the important functional contribution of calnexin to the biogenesis and surface expression of the MHC class I [23]. However, other experiments using human calnexin deficient cell lines showed that calnexin is not absolutely required for class I folding, peptide loading, export and surface expression which suggested that in human cells additional ER chaperones can replace calnexin when its expression is impaired [30].

1.2.2 ERp57

ERp57 (also known as GRP58, PDIA3, ERp60) is a soluble member of protein disulfide isomerase family which resides mainly in the endoplasmic reticulum [31]. ERp57's function is to assist with protein folding in the ER through the formation, breakage and rearrangement of disulfide bonds. In contrast to other PDI family members, ERp57 assists exclusively with glycosylated protein folding by interacting with calnexin and calreticulin [32, 33, 34]. ERp57 contains four well conserved thioredoxin-like domains named a, b, b' a' with the and a' containing the CXXC catalytic motifs responsible for the protein redox activity. The N-terminal cysteine of the active site forms a covalent mixed disulfide bond with its substrates, whereas the C-terminal cysteine of the active site attacks the disulfide bond, facilitating the escape pathway which releases ERp57 from bonds with its substrates [35].

The function of ERp57 was studied extensively due to its role during the assembly of the class I heavy chain and for its role in the peptide loading complex. In the early biogenesis of the heavy chain, ERp57 is recruited by calnexin to catalyze disulfide bond formation within the heavy chain, whereas during the later stages of MHC class I assembly, ERp57 enters the peptide loading complex where it is exclusively conjugated to another ER chaperone, tapasin. Importantly, unlike other ERp57 substrates, the tapasin–ERp57 interaction is further maintained by non-covalent bonds which supports the heterodimer stability [36, 37]. The ERp57– tapasin covalent interaction is mediated by a disulfide bond that requires the N-terminal Trx domain of ERp57 and is formed by the conserved Cys57 of ERp57 and Cys95 of tapasin [38, 39]. Depending on the cell type, this interaction sequesters 15–80% of the total ERp57 pool and also allows the entry of tapasin to the PLC only when it is conjugated to ERp57 [6]. The important role of ERp57– tapasin conjugate in the peptide loading complex was revealed through mutagenesis studies that showed mutation of cysteine 95 of tapasin not only abolishes the recruitment of ERp57 to the peptide loading complex but also impairs the full oxidation of the class I heavy chain that results in poorly stabilized MHC class I on the cell surface [40]. Further support for the critical role of ERp57 during MHC class I assembly came from studies using a B cell line deficient in ERp57. In these cells, the surface expression of the MHC class I was decreased and the normal turnover of the MHC class I was accelerated [41].

1.2.3 Tapasin

Tapasin is a 48 kDa protein encoded by a gene linked to the MHC class II region. It is a member of the immunoglobulin (Ig) super family that contains an immunoglobulin constant domain, a transmembrane domain and a cytoplasmic domain with ER retrieval sequence [42]. Like other antigen presentation genes such as TAP and the LMPs, tapasin expression is induced by interferons (IFNs) [43]. Importantly, unlike other chaperones that associate with many proteins during their folding in the ER, tapasin is solely dedicated to the MHC class I assembly process [8]. Co-immunoprecipitation studies have demonstrated that tapasin is part of the PLC. Since the independent association of tapasin with the class I and TAP was observed in the absence of TAP and class I respectively, it was suggested that tapasin forms a structural bridge between TAP and the remaining peptide loading components [44]. Mutagenesis studies revealed that there are at least two sites of interaction of tapasin with the class I heavy chain mediated through a primary interaction of the N terminal domain of tapasin with the $\alpha 2$ domain of the class I heavy chain [45, 46] with a secondary interaction between the membrane proximal immunoglobulin domain of tapasin with the $\alpha 3$ domain of the heavy chain [47].

The binding of tapasin to TAP on the other hand involves the interaction between the C-terminal transmembrane domain of tapasin with the N-terminal transmembrane domain of TAP [48]. The essential function of this protein was elucidated using a human B cell line deficient in tapasin and knockout mice which exhibited reduced levels of MHC class I and impaired CTL response. This phenotype could be restored upon transfection of a full length tapasin gene, which demonstrated the direct contribution of tapasin to the knockout phenotype [42]. One of the first proposed functions of tapasin came from its direct binding to TAP which suggested its role in stabilzing TAP, increasing the peptide flow to the ER and in improving the peptide loading by recruiting the MHC class I molecule to TAP. However, these modes of action were brought into question when a soluble version of tapasin that could bind the MHC class I but could not bind TAP successfully rescued antigen presentation in cells lacking tapasin expression [49]. Other studies focused on the role of tapasin in quality control and suggested that tapasin plays a role in retaining unstable MHC class I in the ER [50]. Importantly, perhaps the most fascinating and attractive function of tapasin that has been investigated so far is its role as a peptide editor, responsible for optimizing the selection of peptides over time by displacement of peptides with low binding affinity from the peptide binding groove in favour of high affinity ones [8, 51].

1.3 Viruses interference in the PLC

The important role of the peptide loading complex during viral infection is supported by the presence of immune evasion proteins encoded by viruses which target molecules in the peptide loading complex to escape from the consequences of the immune response. This viral strategy results in decreased cell surface levels of MHC class I carrying viral peptides or reduced flow of peptides into the ER both of which ultimately impair recognition of viral peptides by CD8+ T cell lymphocytes [52]. For example ICP47 encoded by herpes simplex virus type 1 (HSV-1) was shown to prevent peptide transport into the ER by blocking the TAP peptide binding site or by destabilizing TAP [53, 54]. Another viral protein which has been shown to target TAP in a different manner is the US6 transmembrane glycoprotein encoded by the human cytomegalovirus (HCMV). This protein was shown to inhibit ATP binding to TAP preventing the translocation of peptides into the ER [55, 56]. Another molecule in the complex that is targeted by viruses is tapasin. E19 is an adenovirus ER protein which has been shown to abolish the interaction between the MHC and tapasin and therefore impair the entry of the MHC class I into the peptide loading complex [57]. Interestingly, this effect was only observed in MHC class I alleles which were dependent on tapasin binding, as a few other MHC class I alleles could reach the cell surface with no interruption [58]. Another protein which interferes with tapasin function in the complex is US3, an ER glycoprotein encoded by the HCMV, which binds to tapasin and is suggested to interfere with its peptide editing function [59].

1.4 MHC in fish

The first genomic MHC fragments in fish were isolated in carp in 1990 [60] and since then the isolation of both MHC class I and class II sequences in many other jawed fish species including sharks was successfully pursued [61, 62, 63, 64, 65]. In trout and salmon a classical class I heavy chain gene is believed to be encoded by one major polymorphic locus named UBA [66], however other loci have been described [67]. Shortly after the identification of the MHC class I heavy chain, the isolation of β2m genes was successfully pursued[68, 69]. In rainbow trout, β2m is encoded by three linked genes of which only two are expressed [70]. Similar to the MHC genes in mammals, fish MHC are polymorphic [71, 72, 73, 74] and expression of these molecules in fish tissues as well as in cell lines mostly follow the same expression profiles [75, 76, 77, 78, 79]. In addition, certain MHC alleles have

been correlated with increased disease resistance [80] as has been seen in other vertebrates [81, 82]. Interestingly, genomic linkage studies revealed a surprising organization in which the fish MHC regions I, II and III do not reside on the same chromosome and hence do not from a complex, therefore they are referred to as Major histocampatibility genes (MH) [83, 84]. The proteasomal subunits: LMP2, LMP7, TAP and tapasin (TAPBP.A) were found to be linked to the class Ia locus in contrast to their close linkage to the class II region in mammals [85, 86, 76]. Despite these genomic discrepancies, the fish MHC proteins most likely function in a similar manner to mammalian homologues considering their sequence similarity, expression pattern and polymorphism.

Evidence for the possible function of the MHC emerged from studies using mixed leukocyte reaction and graft rejection in several fish species, which hinted on their possible role in differentiating between self and non-self-antigens [87, 88, 89]. Evidence supporting the notion that antigen processing and trafficking to the cell surface does exist in fish came from a study which showed that membranes from channel catfish APC pulsed with an exogenous antigen, were able to generate proliferation of peripheral blood leukocytes derived from the same fish. These finding also hinted on the possible presentation of the surface antigen by MHC [90]. More direct evidence for the functional similarity between mammalian and fish MHC was obtained as soon as specific antibodies were generated which could demonstrate cell surface association between MHC class I heavy chain with β2m in catfish B cells [91].

1.5 Regulation of the MHC during viral infection

The transcription of the MHC class I and genes associated with antigen presentation is enhanced during viral infection by the action of interferon (IFN), which plays a major role in antiviral immunity. Interferon belongs to a family of cytokines that in mammals is divided into three classes: type I IFNs (IFNα/β), type II IFN (IFNγ and type III IFNs (including IFNλ1/2/3). The interferon response is triggered by double stranded RNA (dsRNA) generated by most viruses during their replication cycle which is bound by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and retinoic acid-inducible gene I (RIG-I) like receptors [92].

Binding of the interferons both on non-immune and immune system cells initiates a signalling cascade that involves Jak (Janus activated kinase) and STAT (single transducer and activator of transcription) proteins mediating the transcriptional activation of many

interferon stimulated genes (ISG) [93, 94] such as the MHC class I pathway genes.

The isolation of fish interferon genes has been successful in several fish species [95, 96, 97]. Studies in salmonids have shown the induction of the mRNAs of the MHC class I pathway genes, which includes the MH class I HC, β2m, TAP, tapasin and the inducible proteasome subunits LMP2 and LMP7 during *in vivo* infection with infectious hematopoietic virus (IHNV) and during *in vitro* infection with infectious salmon anemia virus (ISAV). Promoter analysis of these genes indicated a putative interferon regulatory factor (IRF) binding site and a IFNγ binding site (GAS) supporting their transcriptional activation by the interferon response [98, 99]. In contrast to mammals, fish are poikilothermic that need to adjust constantly to temperature fluctuations in their environment which can alter their physiology as well as their immune response. Importantly, a study using a murine cell line maintained at low temperature (26°C) showed that the MHC class I molecules can reach the cell surface, however they lack peptides and therefore fail to elicit cytotoxic T cell responses at this temperature [100]. A subsequent study performed in carp maintained at the low end of their permissive temperature range (6°C) showed that expression of the MHC class I molecule in peripheral blood leukocytes is completely abrogated at this temperature. However when fish returned to a more moderate temperature of 12°C, normal levels of MHC class I expression on the cell surface returned [187]. Another study examined the effect of cold temperature on the cellular expression of β2m in salmonid fish and showed that at 12°C the cellular and cell surface levels of this protein are maintained [101]. However, no studies have been conducted so far in fish to determine the influence of cold temperature on the expression of the MHC class I during viral infection.

1.6 Goals of this work

The overall goal of this work is to better understand the fish immune system by characterizing the mechanisms involved in antigen presentation in fish. The core of this study is to identify the peptide loading complex in fish, using rainbow trout as our model species. Five proteins are investigated in this study: ERp57, tapasin, calnexin, class I heavy chain and β2m, which are known to be part of the PLC in mammals.

First, trout ERp57 and calnexin full length sequences are identified and characterized. Then the regulation of tapasin, class I heavy chain and β2ms investigated in RTS11 cells upon exposure to immune stimulation and during *in vitro* infection at cold temperatures with two fish viruses: chum salmon virus (CSV) and viral hemorrhagic septicemia virus

(VHSV IVa). Secretion of β2m into the media of cell cultures was used as a proxy for MH class I turnover and the effect of viral infection at various temperatures on this process was investigated. Subsequently, the putative interactions of the characterized proteins with the MH class I heavy chain was studied using *in vitro* infection with VHSV as a stimulus.

Chapter 2

The cloning and inducible expression of the rainbow trout ERp57 gene.

2.1 Introduction

ERp57 (also known as ER-60/GRP58/PDIA3) is a member of a protein disulfide isomerase family [31]. This protein is located mainly in the endoplasmic reticulum (ER) and has an important role in the correct folding of newly synthesized glycoproteins by mediating the formation of disulfide bonds. This role is carried out through a non-covalent interaction of ERp57 with the ER lectin binding chaperones calnexin and calreticulin, which bind to monoglucosylated oligosaccharides as part of the quality control machinery in the endoplasmic reticulum. ERp57 is also called GRP58 (Glucose regulated protein 58) due to its increased expression levels after glucose depletion [31]. ERp57 protein sequences are highly conserved in vertebrates, invertebrates and in plants which suggests conserved and important biological roles for this molecule. ERp57 contains four thioredoxin (TRX) like domains a,b,b' and a' with two redox CXXC motifs, one in each of the a and the a' domains [34].

One characteristic function of ERp57 is in the assembly of the major histocompatibility complex (MHC) class I in the ER. Early in the biogenesis of the MHC class I heavy chain, ERp57 associates with calnexin and catalyzes the formation of disulfide bonds in the heavy chain thereby facilitating the interaction of the heavy chain with the soluble $\beta 2$ microglobulin [6, 38, 39]. In addition, ERp57 was found to be a part of the peptide loading complex which is composed of several unique functional proteins such as TAP, tapasin, calreticulin

and β2 microglobulin. The covalent and stable association of ERp57 with tapasin seems to help stabilize the peptide loading complex [36]. ERp57 gene knockouts are embryonic lethal at 13.5 days post coitus in mice. Interestingly, deletion of the ERp57 gene in B cells of mice results in normal B cell development [41]. Nevertheless, ERp57 deficient B cells show reduced expression levels of MHC class I on the cell surface and decreased antigen presentation, indicating that ERp57 contributes both qualitatively and quantitatively to MHC class I antigen presentation *in vivo*. Work with both mammals and fish suggests additional roles for ERp57. Despite its ER retention signal, mammalian ERp57 can be found in many cellular locations such as the cell surface, nucleus and cytosol [102], suggesting additional cellular functions. For example, ERp57 is expressed on the surface of the sperm heads where it appears to assist in sperm-egg fusion [103] and participates in the STAT3 signal transduction pathway in human hepatoma cells [104].

In the case of fish much less is known. However, the ERp57 gene has been cloned from the atlantic salmon liver and been shown to change expression after different stresses, leading to the suggestion that it acts as a key link between oxidative and ER stress pathways [105]. To date no studies on the role of ERp57 in teleost fish have been conducted because, while there are some sequences in databases, the gene has not been studied in any detail. Given its role in antigen presentation, understanding the features and function of ERp57 in teleost fish will be important for understanding how teleost fish carry out that process and will perhaps allow the design of fish specific vaccines that better exploit that process. In order to further characterize the ERp57 gene in teleosts and to begin exploring its roles in the immune system, a full length cDNA clone was isolated from rainbow trout, our model species, and analyzed in terms of its tissue distribution as well as its expression pattern under *in vivo* and *in vitro* immune and ER stress stimulating conditions.

2.2 Material and methods

2.2.1 Fish

Rainbow trout (100–180 g) were obtained from Silver Creek Aquaculture (Erin, ON) and kept in 200 L fresh-water flow-through tanks at the University of Waterloo under 12:12 h light-dark cycle. Water temperature was maintained continuously at 13°C. Fish were fed 5 days/week with commercial dry pellet (5 point floating trout chow from Martin Mills, Elmira, ON). Animals were kept according to the Canadian Council on Animal

Care (CCAC) guidelines under the approval of the University of Waterloo Animal Care Committee.

2.2.2 Cloning of rainbow trout ERp57 cDNA

Based on a multiple sequence alignment of *Homo_ sapiens* [NP 005304.3], *Mus musculus* [NP_ 031978.2], *Canis familirais* [XP_ 535453.2], *Rattus norvegicus* [NP_059015.1] and *Danio rerio* [XP_002665576.1], conserved regions of the ERp57 protein were located and degenerate primers were designed. Total RNA was extracted from $1{\times}10^7$ RTS11 cells using Trizol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Subsequently, single strand cDNA was generated using a Fermentas RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific, Nepean, ON) using 50 ng of oligo$(dt)_{18}$ and RevertAid M-MuLV Reverse Transcriptase according to manufacturer's instructions.

The PCR reaction mix (25 μL) contained 1 μg of RTS11 cDNA, 1x PCR buffer, 200 μM dNTP mix, 1 mM MgCl2, 1U of Taq Polymerase (MP Biomedical, Solon, OH) and 600 nM of both sense primer 5' TGGTGYGGNCAYTGYAAR 3' and antisense primer 5' NGCRTCCATYTTNGC 3'. The reaction parameters were 95°C for 5 min and 30 cycles of 95°C 40 sec, 48°C 1 min, 72°C 1 min with a final extension for 15 min in 72°C using a BioRad DNA Engine thermocycler (BioRad, Mississauga Ontario).

The 1000 bp amplicon which was excised, purified and ligated into pGEM-T Easy vector (Promega,Madison, WI) was subsequently transfected into XL1-Blue MRF' *E.coli* competent cells. Plasmids containing the insert were sequenced at the Center of Applied Genomics (Toronto, Ontario).

2.2.3 Generation of full length cDNA using RACE

In order to extend the cDNA ends of the ERp57 partial fragment both 5' and 3' RACE were performed using a method adapted from Frohman et al.[106]. 5 μg of total RNA derived from RTS11 was isolated as previously described and combined with 50 ng of oligo$(dT)_{13}$ primer sequence containing *Hin*d III, *Sst*I, *Xho*I recognition sites as follows: 5' CAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCTTTTTTTTTTTTT 3'. 20 μL of Single Strand cDNA synthesis mixture was generated as previously described. Reverse transcription mixtures were incubated for 60 min at 42°C, 10 min at 50°C, and 15 min at 65°C. Subsequently, 1.5 U of RNAseH (Invitrogen, Carlsbad, CA) was added to the

reaction mixture, which was incubated at 37°C for 20 minutes. The mixture reaction was diluted to 1 mL with Tris–EDTA solution (10 mM Tris-HCl pH [7.5], 1 mM EDTA pH 8.0). The PCR reaction mix was made as previously described using 3 μL aliquot of the cDNA 3' RACE pool with gene specific sense primer (GSP1) 5' ACTTGACGGCAGCTGAAGCG 3' and RACE anti sense primer 5' CTTGCTGACGGTGGAAGCCCAGC 3' to a final volume of 25 μL. PCR parameters for the first cycle were as follows: 5 min at 95°C, 2 min at 54°C and 30 min at 72°C. Next 30 cycles were executed at 94°C for 15 sec, 54°C for 30 sec and 72°C for 4 min. The PCR products were diluted 1:10 and used for subsequent nested PCR with an internal gene specific primer (GSP2) 5' TGCCACAGCCAATGACGTGC 3' and RACE primer 5' GAGGACTCGAGCTCAAGC 3'. Reverse transcription for 5' RACE was performed as previously described using an antisense primer 5' TCCACAGTGTTCGCAGAGCG 3' specific to ERp57.

The reaction mixture was diluted to 400 μL with Tris–EDTA buffer (10 mM Tris-HCl pH [7.5], 1 mM EDTA pH 8.0) purified to a final volume of 12 μL using PCR purification kit (Qiagen,Mississauga, ON) according to manufacturer's instructions. Tailing buffer (1X) (Invitrogen, Carlsbad, CA) was combined with 4 μL of 200 μM of dATP and 10U of terminal deoxynucleotidyl transferase (Invitrogen, Carlsbad, CA). The reaction mixture was incubated for 15 min at 37°C following 5 min of inactivation at 65°C for 10 min. A PCR reaction was performed as previously described using gene specific primer 5' CTTGGCAGTGCGGATGCCGA 3' with RACE primer 5' CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCTTTTTTTTTTTTT 3' reaction parameters were similar to 3' RACE except for the first cycle as follows: 95°C for 5 min, 48°C for 2 min and 72°C for 40 min.

2.2.4 Southern blot analysis

Genomic DNA was isolated from rainbow trout peripheral blood leukocytes using a Wizard Genomic DNA purification kit (Promega, Madison, USA). Ten μg of genomic DNA was digested using *Eco*RV,*Hin*dIII, *Kp*nI and *Pst*I (ThermoFisher Scientific, Nepean, ON). The digests were electrophoresed on a 1% agarose gel and transferred to a positively charged membrane (Roche, Manheim, Germany), followed by UV cross linking for 45 sec. After 4 hr of prehybridization in DIG hybridization granules (Roche, Manheim, Germany), the membrane was hybridized at 42°C for 16 hr with 446 bp DIG labelled cDNA probe. The probe was prepared by using DIG synthesis kit (Roche, Manheim, Germany), ERp57 specific primers 5' TTGACGGCAAGCTGAAGCG 3' and 5' CTAGTGATTGAAGCTTC-

TATAGCTC 3' and the full length rainbow trout ERp57 cDNA plasmid clone as template. The membrane was washed once with 0.5X SSC for 15 min and twice at 65°C for 30 min in 0.1X SSC containing 0.1% SDS. Hybridized DNA fragments were detected as using CDP star and anti-DIG antibody conjugated to alkaline phosphatase according to manufacturer's protocol (Roche, Manheim, Germany).

2.2.5 Phylogenetic tree construction and sequence analysis

Nucleotide sequences were obtained from the GeneBank database and are listed in the table 2.1.

Species	**Accession number**
H. sapiens	NM_005313.4
P. troglodytes	XM_001159572.2
B. taurus	NM_174333.3
C. lupus	XM_535453.3
M. musculus	NM_007952.2
R. norvegicus	NM_017319.1
G. gallus	NM_204110.3
S. salar	BT_044896.1
S. salar	NM_007952.2
O. mykiss	EZ_774749
D. rerio	NM_001199737.1
S. salar	BT046042.1
S. salar	BT058988.1
D.melanogaster	NM_165849.2
C. elegans	NM_059594.5
O. sativa	BT058988.1
S. salar	NM_001053654.1

Table 2.1: ERp57 nucleotide sequences obtained from the GeneBank database.

The tree was constructed using neighbour joining method with bootstraps values through 1000 replications. Numbers above the line indicate the percent bootstrap confidence values. Signal peptide of trout ERp57 was identified using SignalP 3.0 server. Protein conserved domains were identified using the NCBI conserved domain (CDD) platform.

2.2.6 Construction of ERp57 expression vector

The full coding region of ERp57 was amplified using PCR primers designed to contain both *Bam*HI and *Hin*dIII sites in the sense primer 5' GGATCCATGTTGAAATTGTTTTT 3' and antisense primer 5' GAAGCTTCTATAGCTCAATCTGAATATTCTTCTT 3' respectively using RTS11 cDNA template. The PCR parameters were as follows: 95°C for 5 min, 35 cycles of 94°C for 40 sec, 53°C for 45 sec, 72°C for 1 min and 50 sec and final elongation at 72°C for 10 min. The amplified 1496 bp PCR product was ligated into pGEM T easy (Promega, Madison, USA) and was sequenced at the Center for Applied Genomics (Toronto, Ontario, CA). The insert was excised using *Bam*HI and *Hin*dIII (Fermentas, Burlington, CA) at 37°C for 2 hr and purified using a gel extraction kit (Qiagen, Mississauga, ON). The pRSET A expression vector (Invitrogen, Carlsbad, CA, USA) was digested using the same restriction enzymes and purified as previously described. The purified coding sequence of ERp57 was ligated to pRSET A using T4 ligase (Promega, Madison, USA) overnight at 4°C. The constructs were transformed in BL21 (DE3) PlysS (Invitrogen, Carlsbad, CA, USA) and were sequenced to verify that the inserts were in the correct frame with 6XHIS and Xpress epitope N-terminal tags.

2.2.7 Protein production and purification

A 50 mL of overnight culture grown at 37°C was used to inoculate 500 mL LB broth. The cells were grown until the OD600 reached 0.4-0.6 and then induced with 1 mM of isopropyl–beta–D–thiogalactopyranoside (IPTG). Following induction, the cells were grown for another 5 h. The cells were harvested by centrifugation at 10,000 rpm for 10 min. Protein was purified using Ni-NTA Agarose (Qiagen, Mississauga, ON) according to manufacturer's instruction. Briefly, one mL of Ni-NTA Agarose resin was added to 10 mL of cleared cell lysate and incubated for 1 hr at 4°C. After incubation, the lysate was loaded onto a 25 mL econocolumn (Bio-Rad Laboratories, Mississauga, ON) and washed with binding buffer (100mM NaH2PO4, 10mM Tris, 8M urea) equilibrated to pH 8.0, pH 6.3, and pH 5.9. Finally recombinant ERp57 was eluted at pH 4.5 and was stored at 4°C The elution fractions were separated on a 15% sodium dodecyl sulfate (SDS) gel electrophoresis. Following SDS-PAGE, the proteins were transferred onto a nitrocellulose membrane blocked with 5% skim milk in T-TBS buffer (0.14 M NaCl, 2.7 mM KCL, 25mM Tris, 0.5% Tween 20, pH 8) for 1 hr. The membrane was washed with T-TBS for 5 min and probed with 1:2000 mouse anti-express antiserum (Invitrogen, Carlsbad, CA, USA) to identify the specific Xpress

epitope tag on the recombinant protein.

2.2.8 Production of polyclonal antibodies

Two New Zealand white rabbits (Charles River, ON, CA) were injected intramuscularly with an initial dose of 0.5 mg of rERp57 with Freund's complete adjuvant (1:1). A boost using 0.5 mg recombinant ERp57 with Freund's incomplete adjuvant was given every three weeks. Rabbits were exsanguinated after 12 weeks and approximately 50 mL of serum was collected from each rabbit. Ear bleeds were performed every three weeks to verify the antibody titer. The specificity of the antibody was demonstrated in the following way. Extracts from rainbow trout tissues were prepared as described in section 2.3.12, run on SDS PAGE gels and transferred to nitrocellulose membranes for western blot analysis. Nitrocellulose membranes with transferred proteins from various tissue extracts were incubated with or without ERp57 recombinant protein (0.5 mg/mL) added to the polyclonal antiserum. When the recombinant protein was absent, bands around 57 kDa were detected (see results Figure 2.3.4). This is higher than the 53 kDa expected molecular weight based on the amino acid sequence, but normal for ERp57 as its name suggests. No bands were detected when ERp57 recombinant protein was present. Therefore, the antibody was specific for ERp57.

2.2.9 Rainbow trout cell lines

The rainbow trout cell lines were the monocyte/macrophage cell line, RTS11 [107], and the epithelial-like cell lines: RTL-W1 from a normal liver [108] and RTgill-W1 from the gill [109]. All cell lines were grown at 20°C to 22°C in L-15 with 15% of fetal bovine serum (FBS) as described [79].

2.2.10 Tissue collection of rainbow trout

Fish were anesthetized in 1 mL/Liter of 2-phenoxyethanol (Sigma Aldrich St. Louis, MO). Blood was drawn from the caudal vein of rainbow trout using 22G needle using a syringe prefilled with 100 units of heparin (Sigma Aldrich, St. Louis, MO) in 0.85% NaCl solution. Tissue samples were collected from the head kidney, thymus, liver, brain, heart, gill, spleen, muscle, hind gut, ovary and ovulated eggs. Tissues were either resuspended in PBS for

western blotting or in RNAlater (2.5 mM Na citrate, 5.3 M $(NH_4)_2SO_4$, 0.01 M EDTA, pH 5.2) for RNA extraction.

2.2.11 Northern blot analysis

RNA was isolated from rainbow trout tissues using Trizol according to manufacturer's protocol (Invitrogen, Calsbad,CA, USA) and treated with DNase I, RNase-Free for the removal of any genomic DNA (Qiagen, Mississauga, ON), according to the manufacturer's instructions. The RNA was dissolved in PCR grade water (Roche, Manheim, Germany) and stored at -80°C until used. Six μg of total RNA was separated on a 1% agarose-formaldehyde gel and transferred to a positively charged membrane (Roche, Manheim, Germany), followed by a UV crosslinking. The membrane was prehybridized with DIG hybridization granules (Roche, Manheim, Germany) for 4 hr following hybridization at 50°C for 16 hr [110] with the same cDNA probe as previously described in (2.2.4). The bands were detected using alkaline phosphatase conjugated anti-DIG antibody and CDP star (Roche, Manheim, Germany) using BIS 303 PC (Montreal Biotech Inc., Dorval, PQ, Canada).

2.2.12 Western blot analysis

For protein extraction, 80 mg of tissue was added to 300 μL NP-40 lysis buffer (1% (v/v) NP-40, 150 mM NaCl, 50 mM Tris [pH 8.0]) supplemented with 1X of protease inhibitor cocktail (Roche). Tissue lysates were sonicated on ice in short bursts and crude lysates were cleared by centrifuging at 4°C for 15 min at 13,000 rpm. Supernatants were collected and quantified for protein concentration using bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Nepean, ON) using manufacturer's protocol. Tissue lysates (50 μg) were mixed with 5x Laemmli sample buffer and boiled for 10 minutes prior to loading. Samples were separated on a 12% acrylamide gel and transferred to nitrocellulose membranes (Bio-Rad, Mississauga, ON) and subsequently blocked with 5% skim milk in TBS-T (0.14 M NaCl, 2.7 mM KCl, 25 mM Tris, 1% Tween 20; pH 7.5) for 1 hr. Blots were probed with primary antibody for 1 hr using ERp57 antisera diluted 1:1000. The membrane were washed with TBS-T three times 10 min each then the secondary antibody, goat anti Rabbit alkaline phosphatase (Sigma Aldrich St. Louis, MO), was applied in a 1:30000 dilution and probed for 1 hr. Bands were detected using NBT/BCIP (Roche, Manheim, Germany) according to manufacturer's instructions.

2.2.13 Induction of ERp57 in rainbow trout *in vivo*

Two rainbow trout were injected intraperitoneal with phytohemagglutinin PHA-M (Sigma Aldrich, St. Louis, MO) at a final concentration of 50 mg/250g of fish in L-15 medium and sacrificed after 48 and 96 h. Two fish were used as controls and were injected with an equivalent volume of L-15 media vehicle control only. Following dissection, tissue was collected from head kidney, spleen, thymus, gill, heart muscle and brain and store in -20°C in RNA later.

2.2.14 Induction of ERp57 in rainbow trout cells *in vitro*

The induction ERp57 was studied in the RTS11 macrophage-like cell line and in primary culture of peripheral blood leukocytes (PBL). Two classes of potential inducers, poly I:C and A23187, were examined. The synthetic dsRNA and viral mimic, poly I:C, was studied only in RTS11. The calcium ionophore A23187 was studied in both culture systems. While the carrier, DMSO, might have slight effects, the effects of the above agents were the most interesting and these were studied by comparing the treated samples to the carrier alone samples.

In vitro poly I:C stimulation

For exposure to poly I:C, RTS11 (2×10^6) were seeded overnight in a $25\,cm^2$ culture dish with L-15 media supplemented with 150 U/ mL of penicillin and 150 mg/mL streptomycin in 15% fetal bovine serum (FBS). The culture was treated with 50 μg/mL of polyinosinic-polycytidylic acid (Sigma Aldrich, St. Louis, MO) in water or with the same amount of filtered water as a control. Cells were harvested after 12, 24, and 48 h. RNA was isolated using RNeasy kit (Qiagen, Mississauga, ON) according to the manufacturers instructions. Reverse transcription was performed as previously described and transcript levels were determined using PCR amplification of the full coding sequence of ERp57 using 5' AGGATCCATGTTGAAATTGTTTTT 3' and antisense primer 5' GAAGCTTC-TATAGCTCAATCTGAATATTCTTCTT 3' respectively and ribosomal protein S11 control primers using sense primer 5' AGCAGCCAACCATCTTCCAG3' and antisense primer 5' ACTCTCCGACGGTAACAATG 3'. Densitometry analysis was performed to measure changes in ERp57 transcript levels using IMAGE J (version 1.44) and adjusted to the values relative to the control gene.

In vitro A23187 stimulation

For the preparation of PBL cultures, blood samples were centrifuged at $200 \times g$ for 10 min, buffy coat was collected and mixed with 45 mL of L-15 medium (Sigma Aldrich St. Louis, MO). The mixture was split into six fractions and each was layered on the top of 3 mL histopaque 1077 (Sigma-Aldrich, St. Louis, MO). The tubes were centrifuged for 30 min at $400 \times g$. The leukocyte layer located at the media:histopaque interface was collected and cells were pelleted by centrifugation at $400 \times g$ for 5 minutes at room temperature. The supernatant was aspirated and the pellet was washed using 1 mL of 1X phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4; pH 7.4). The PBS was aspirated and the cell pellets were resuspended either with Trizol for RNA extraction or 1% NP40 lysis buffer for western blotting. Peripheral blood leukocytes and RTS11 (2×10^6) were seeded overnight in a $25\,cm^2$ culture dish with L-15 media supplemented with 150 U/mL of penicillin and 150 mg/mL streptomycin in 15% FBS. The culture was treated with 2 μM in DMSO of A23187 (Sigma Aldrich, St. Louis, MO) same amount of vehicle was used for the control. Cells were harvested at different time points in 24 hr time course. Protein extraction and western blotting analysis was performed as previously described using 15 μg of total protein in each lane. RNA isolation and reverse transcription was conducted as previously described, using trout gene specific primers as indicated in Table 2.2. The ERp57 primers used above will only detect our ERp57 sequence and not trout EZ774749 nucleotide sequence.

Gene	**Primer Forward(5'-3')**	**Primer reverse(5'-3')**
ERp57	ACTTGACGGCAGCTGAAGCG	TGCCACAGCCAATGACGTGC
ClassII β	AAGCTTATGTCGATGCCAATT	CGGCCGATTCTAGAGCACCCCAGAAGAC
TGFβ	GCTAAGCTTCTAGCTACACCTGCAG	GCAAAGTAGCCCAGTGGGTTC
β2m	TGTCAATCGTTGTACTTGGG	CTTCAGGTGGCGGACTCTGC
S11	AGCAGCCAACCATCTTCCAG	ACTCTCCGACGGTAACAATG

Table 2.2: Primers sequences for reverse transcription.

2.2.15 Statistical analysis

Statistical analysis was performed using one-way ANOVA and Tukeys *post hoc* test in order to determine if there was a significant change of ERp57 protein levels upon *in vitro* stimulation. A probability of $P<0.05$ was considered significant.

2.3 Results

2.3.1 Trout ERp57 clone isolation and sequencing

In order to clone rainbow trout ERp57, degenerate oligonucleotides were designed based on a multiple species sequence alignment. The forward primer included 12 nt spanning the region encoding the CXXC active redox motif in the classical TRX domain shared by all ERp57 homologs. The reverse primer was designed to anneal in the section of the gene encoding distal C terminal TRX domain (a'). An estimated 1100 bp band was isolated from cDNA obtained from the RTS11 cell line. The sequence showed 95% nucleotide identity to atlantic salmon protein disulphide isomerase a, member 3 mRNA (the atlantic Salmon ERp57 homolog). Subsequently, primers were designed for 5' and 3' Rapid Amplification of cDNA Ends (RACE) reactions, yielding an estimated 700 bp and 200 bp fragments for the 3' RACE and 5' RACE reactions respectively. PCR amplification of the full open reading frame of ERp57 was then conducted using 50 and 30 gene specific primers. The resulting full cDNA sequence is 1985 bp, including 111 bp of 5' UTR and a 392 bp of 3' UTR containing a poly (A) signal located 355 bp downstream of the translation stop codon. The coding region is 1482 bp which encodes a putative 477 aa mature protein with a predicted molecular weight of 53 kDa.

The precursor protein is predicted to have a hydrophobic signal sequence by Signal P server. Amino acid sequence alignment revealed that four classic domains are conserved in trout ERp57, which share 75% sequence identity with the human protein sequences. First, the N terminal classic TRX domain a with a redox active CXXC motif. Second and third two redox inactive TRX-like domains b and b' and a fourth C terminal TRX domain a' containing a second CXXC motif (Figure 2.3.1). Interestingly, the teleost sequences do not include ER retention signal that appears as Q/ KEDL in the mammalian c-terminus and only the zebrafish sequence is predicted to contain a nuclear localization signal that appears as lysine rich sequence upstream of the ER retention signal. Post translational modification in human ERp57 such as phosphorylation on serine 150 and tyrosine 444 have been described [111, 112] and amino acids in these locations were found to be conserved in the trout sequence, suggesting that trout ERp57 can be post-translationally modified. This sequence is slightly shorter at 1955 bp, and shares 92% nucleotide identity in the coding region with the trout sequence isolated above. In addition, 5' UTR and 3' UTR alignment between our trout sequence and EZ774749 shows 91% and 78% sequence identity respectively.

Importantly, the EZ774749 coding sequence uses a different start codon that results in the loss of the N-terminal signal peptide and 20 aa of the functional conserved domain a' in the encoded protein, which both appear to be conserved in our trout and other teleost sequences. In order to further compare our trout nucleotide sequence with trout EZ774749 and additional salmon orthologous sequences, a phylogenetic tree was constructed using ClustalW (Figure 2.3.2) Interestingly, the two rainbow trout sequences are found in separate branches, yet clustering with high confidence with unique salmon sequences. These results suggests that ERp57 has duplicated into two paralogues in the salmonid lineage that share a common ancestral sequence. This gene duplication may be the result of genome duplications that occurred early in salmonid evolution [113].

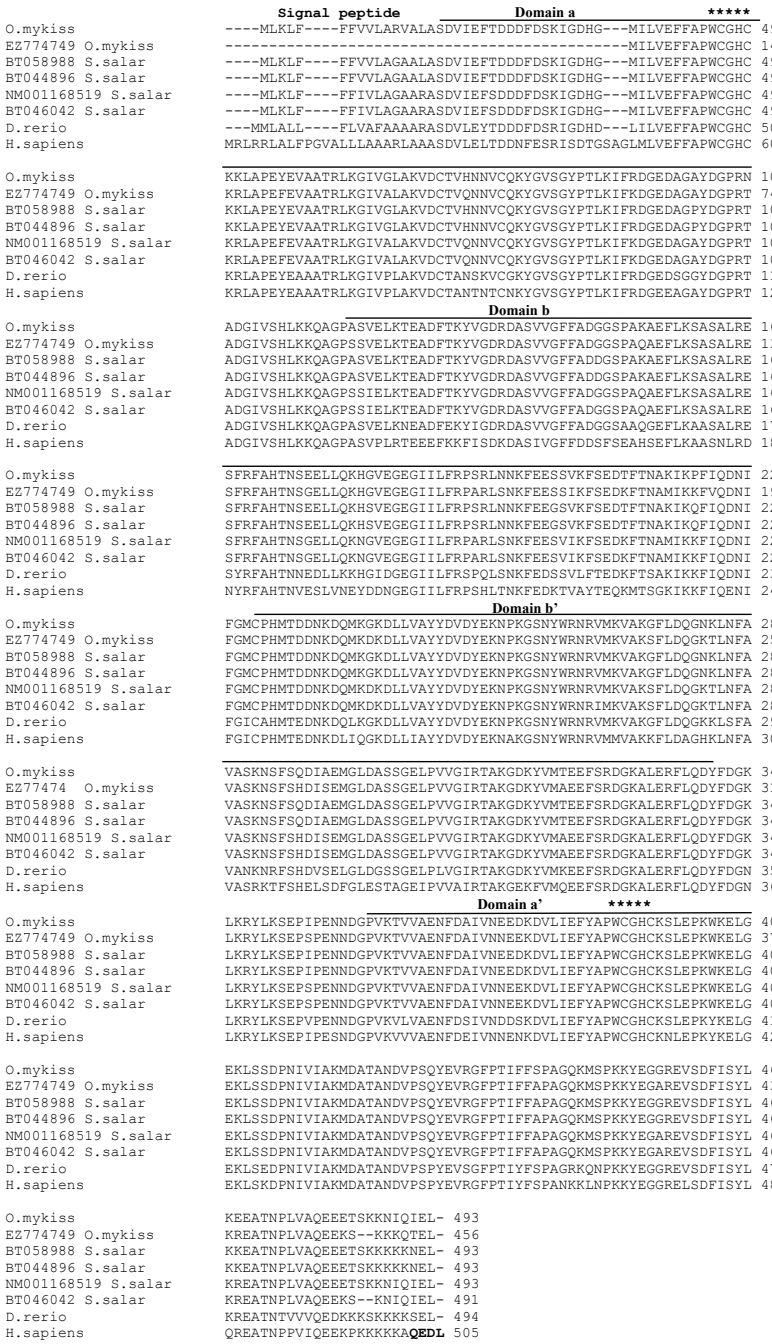

Figure 2.3.1: **Sequence alignment of trout ERp57 with known proteins sequences.**
ERp57 predicted sequences were aligned with the human and teleost fish sequences. Numbers on the right indicate amino acid positions. The four conserved domains are labeled with a black overline: thioredoxin (TRX)–like superfamily N terminal domain (domain a); the first redox inactive TRX-like domain (domain b); the second redox inactive TRX–like (domain b') and the C terminal TRX domain (a'). The redox active CXXC motifs within the sequence are marked with asterisks. The human ER retention signal is marked in bold.

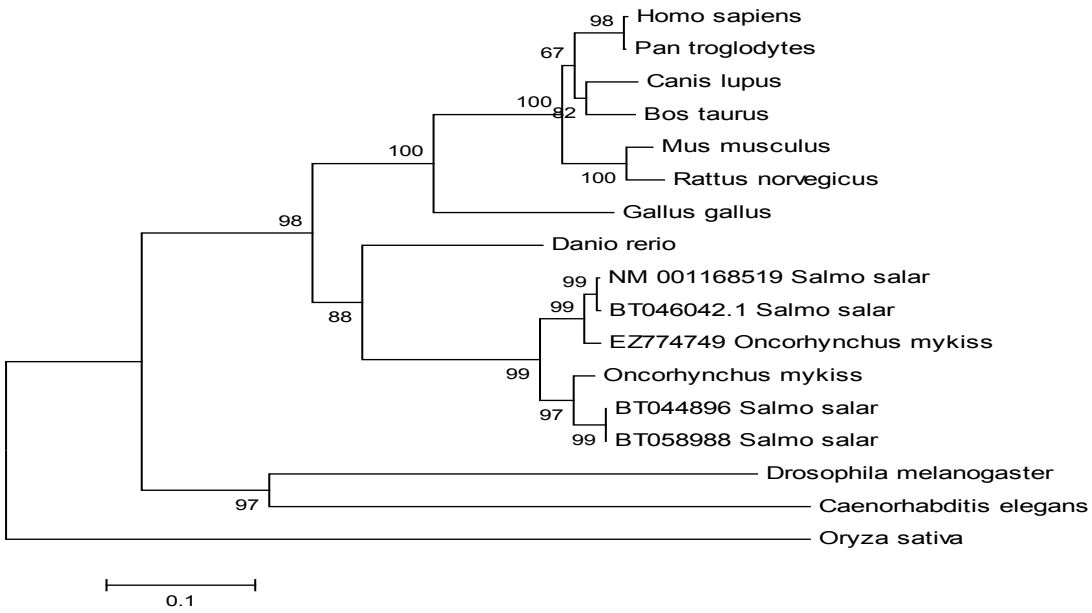

Figure 2.3.2: **ERp57 phylogenetic tree.**

A phylogenetic tree constructed using known nucleotide sequences of ERp57 derived from NCBI homologue gene database. The sequences were aligned using Muscle Aligned sequences were used to construct a phylogenetic tree in MEGA4. The tree was constructed using neighbour joining method with bootstraps values through 1000 replications. Numbers above the line indicate the percent bootstrap confidence values.

2.3.2 ERp57 gene copy number

Southern blotting analysis revealed that there are most likely two copies of ERp57 gene in the trout genome as there are two main bright bands in each digest (Figure 2.3.3). This result supports the phylogenetic tree obtained in Figure 2.3.2. The faint bands in *Pst*I/ *Hin*dIII digest might be due to internal *Pst*I/ *Hin*dIII restriction sites in the gene sequence or hybridization of the probe to different ERp57/PDI family member.

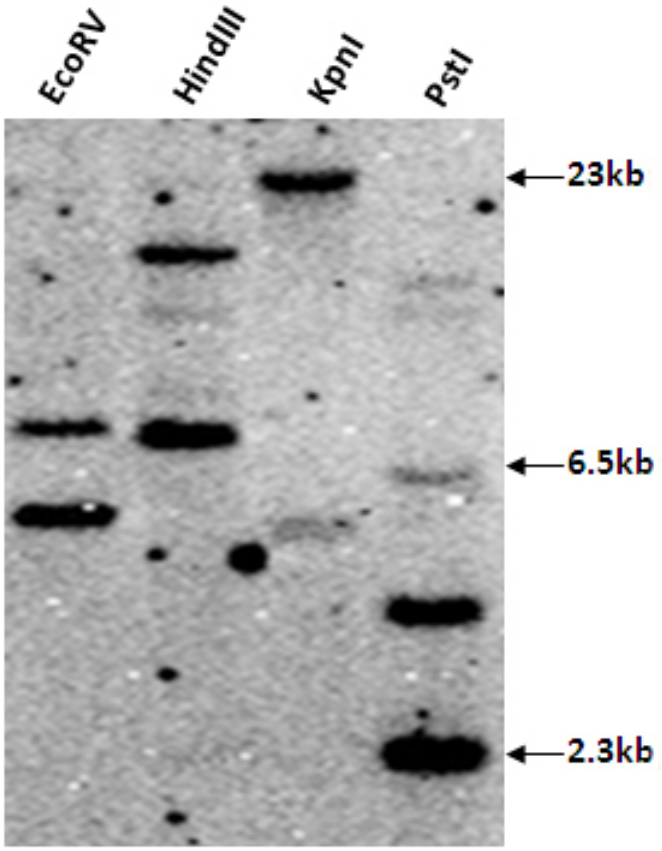

Figure 2.3.3: **Southern blot of trout ERp57.**
Southern blots of rainbow trout ERp57 gene. Ten micrograms of genomic DNA was isolated from rainbow trout peripheral blood leukocytes and was digested using *Eco*RV, *Hin*dIII, *Kp*nI and *Pst*I. Digests were hybridized with a DIG labelled cDNA probe upon transfer to a positively charged membrane. The position of the size markers (λ/*Hin*dIII digest) are shown by arrows on the right margin.

2.3.3 ERp57 transcript expression and protein levels in rainbow tissues and cell lines

At both the transcript and polypeptide levels, ERp57 was expressed in most rainbow trout tissues. Northern blotting showed that the gene encoding ERp57 was expressed strongly in liver and in egg. The high ERp57 expression level seen in the rainbow trout liver correlates with previous findings for the atlantic salmon [105] and human [114]. The trout ERp57 transcript was expressed in all tissues tested with lowest expression in the muscle tissue (Figure 2.3.4A). The most prominent expression of ERp57 protein was also in the liver and egg, which correlates with the highest transcript levels seen in northern analysis. ERp57 protein expression was also shown in the primary immune system organ, head kidney, and in other lymphoid associated tissues such as the spleen and gill (Figure 2.3.4B). The spleen showed the highest ERp57 protein expression levels of the immune related organs. In addition, the protein was expressed in trout cell lines RTS11 and RT gill, with the highest levels found in a trout liver cell line (RT liver), which correlates with the high expression of the transcript and the protein in liver tissue (Figure 2.3.4A and B). Given that the polyclonal antibody should detect the protein products of both our sequence and the one encoded by trout EZ774749, this expression pattern most likely represents a composite of the expression of both forms.

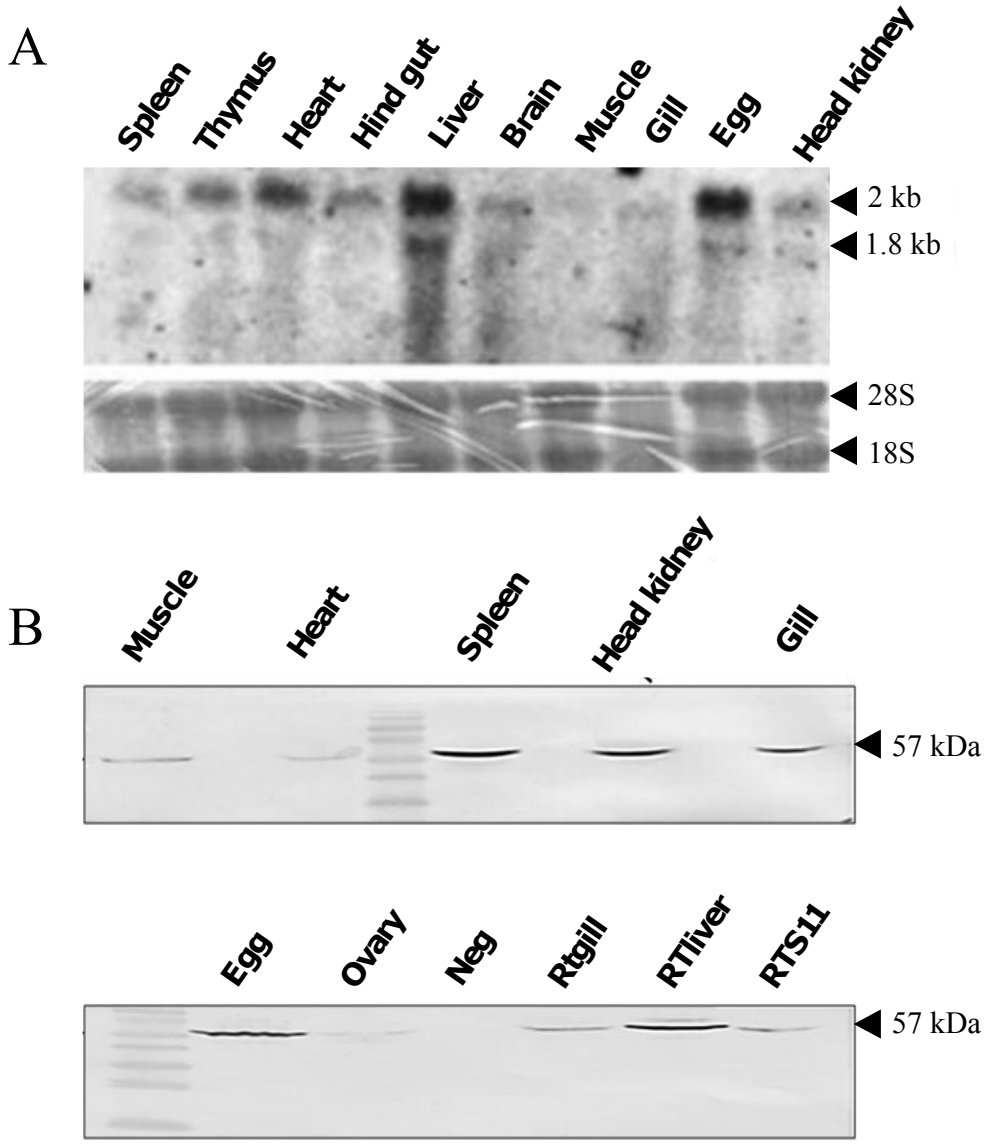

Figure 2.3.4: **Transcript and protein distribution of ERp57 in trout under normal conditions.** A) Northern blot analysis showing tissue distribution of rainbow trout ERp57 mRNA in unstimulated tissues. RNA from trout tissues was separated on a 1% agarose-Formaldehyde gel, transferred to a positively charged membrane and hybridized with 446 bp cDNA DIG labelled probe. The size of the 28S and 18S bands are indicated and shown to indicate the integrity of the RNA. Estimated transcript sizes are indicated with an arrowhead on the right. B) Western blot analysis of ERp57 protein expression in trout tissues and cell lines using polyclonal ERp57 antibody. Expression of trout ERp57 in the egg and ovary is shown in the lower panel. Both tissues and cell lines were used under normal unstimulating conditions. The position of the protein size marker is shown by the arrowhead at the right margin.

2.3.4 Induction of ERp57 in rainbow trout *in vivo*

Induction of ERp57 gene expression was seen in several tissues 48 h after the injection of rainbow trout with phytohemagglutinin (50 mg/250g body weight). Northern blot analysis showed that ERp57 transcript levels increased relative to the control samples 48 and 96 h post stimulus in spleen, gill and heart tissues (Figure 2.3.5). ERp57 expression in the head kidney was undetectable in the control and the 48 h time point although a weak band was observed after 96 h. The difference in the degree and timing of head kidney inducible ERp57 gene expression seen between Figures 2.3.4 and 2.3.5 is probably due to inter-individual variation.

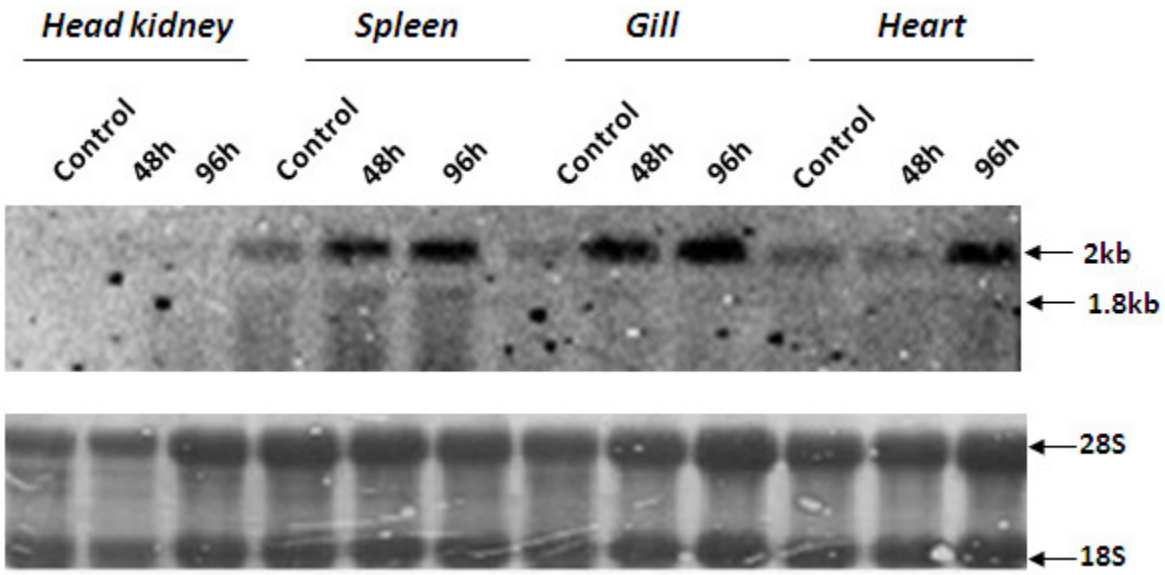

Figure 2.3.5: **Transcriptional upregulation of ERp57 by phytohemagglutinin.**
Northern blots of rainbow trout ERp57 in selected tissues after 48 and 96 hr of in *vivo* injection of two fish with phytohemagglutinin (PHA) at a final concentration of 50 mg/250 g of fish. Two control fish were injected with an equivalent volume of L-15 media only. The size of the 28S and 18S bands (shown in the lower panel) and the estimated transcript sizes are indicated with arrows on the right.

2.3.5 Induction of ERp57 in rainbow trout RTS11 cells *in vitro*

The viral mimic poly I:C, added at 50 μg/mL, induced ERp57 gene expression in the rainbow trout monocyte/macrophage cell line, RTS11, as detected by RT PCR (Figure 2.3.6). When transcript levels were followed over 48 h, ERp57 transcripts were elevated by 12 h but subsequently declined over the next 48 hours.

The calcium ionophore, A12387, which is known to cause endoplasmic reticulum stress [115], induced the expression of ERp57 within 6 h in primary cultures of PBL and in RTS11. In RTS11 cultures ERp57 mRNA levels were upregulated after 6 h of treatment while MHC class II β and β2m mRNA levels didn't change and TGFβ beta levels were downregulated relative to ribosomal protein S11 controls (Figure 2.3.7).

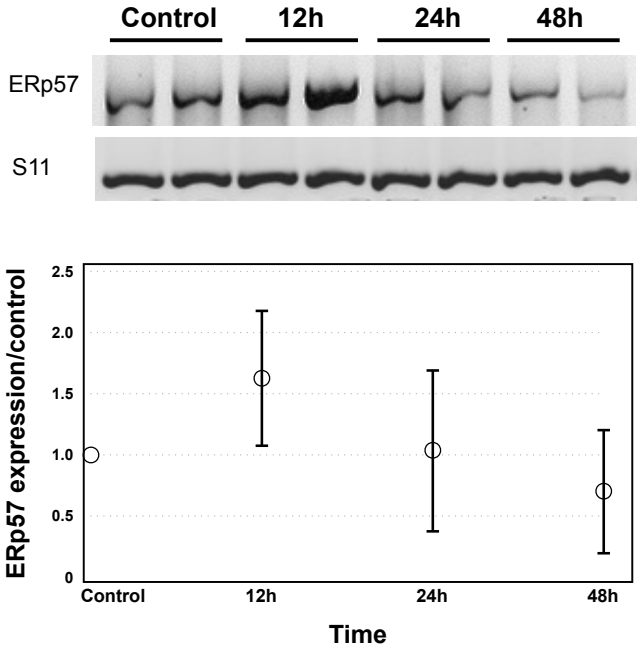

Figure 2.3.6: **Upregulation of ERp57 transcript levels in RTS11 cell line following poly I:C treatment.**
A) RT PCR analysis showing ERp57 and ribosomal RNA S11S11 transcript levels after *in vitro* stimulation with 50 μg/mL of poly I:C during a 48 h time course challenge. Duplicate lanes for each time point represent two separate experiments. B) Densitometry analysis was conducted using image J software (version 1.44) and results are expressed as the ratio between ERp57 and ribosomal protein S11 amplicons that were used as an internal standard for each time point and vertical error bars represent the standard error.

ERp57 protein levels in treated PBL were upregulated up to 3 fold versus the control (data not shown). In the RTS11 cell line upregulation of the ERp57 protein was delayed relative to the upregulation seen in PBL primary culture but increased to nearly 3 fold by 24 h (Figure **??**). In RTS11 culture, DNA laddering was observed after 24 h (data not shown), suggesting that ultimately A23187 induced stress and apoptosis.

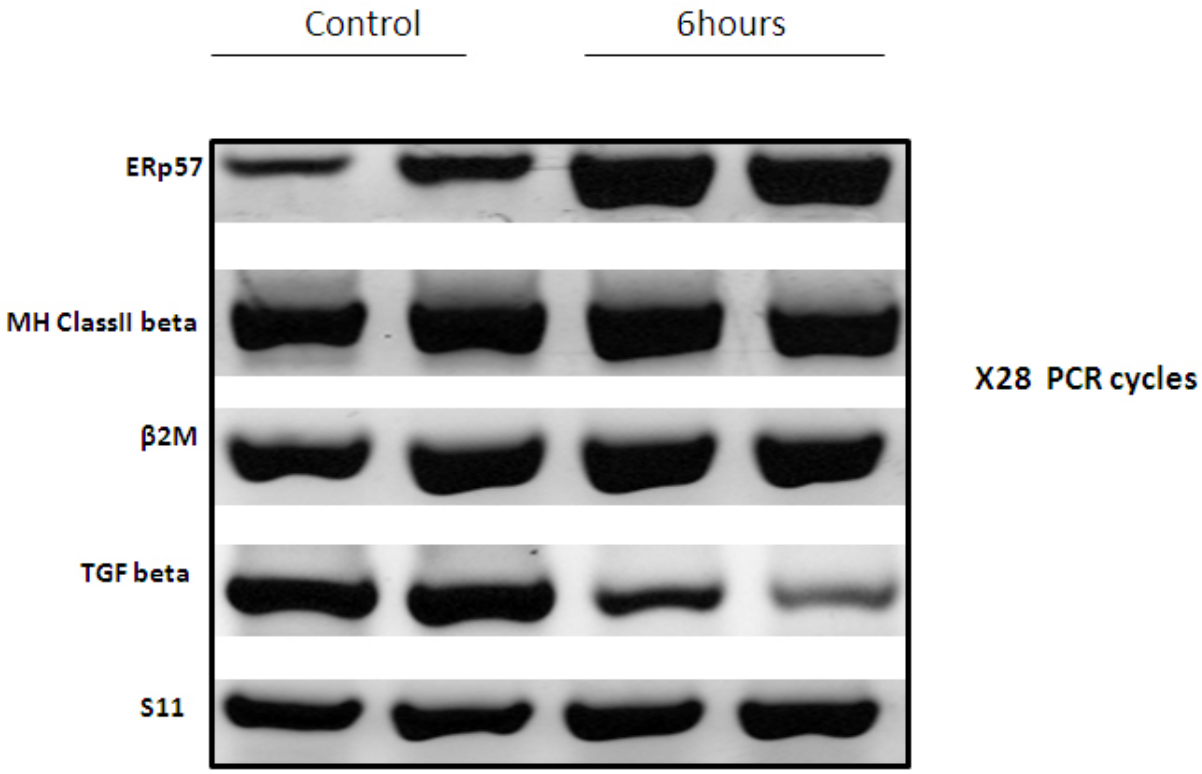

Figure 2.3.7: **ERp57 transcriptional upregulation in RTS11 cell line upon induction with A23187.**

Reverse transcription PCR analysis of ERp57, MH class II beta, β2 microglobulin (β2m), transforming growth factor β (TGFβ) transcript levels in RTS11 cell line after 6 h of *in vitro* stimulation with 2 mM of calcium ionophore A23187. Ribosomal protein S11 transcript bands were used as an internal standard. Duplicate lanes for each time point represent separate experiments.

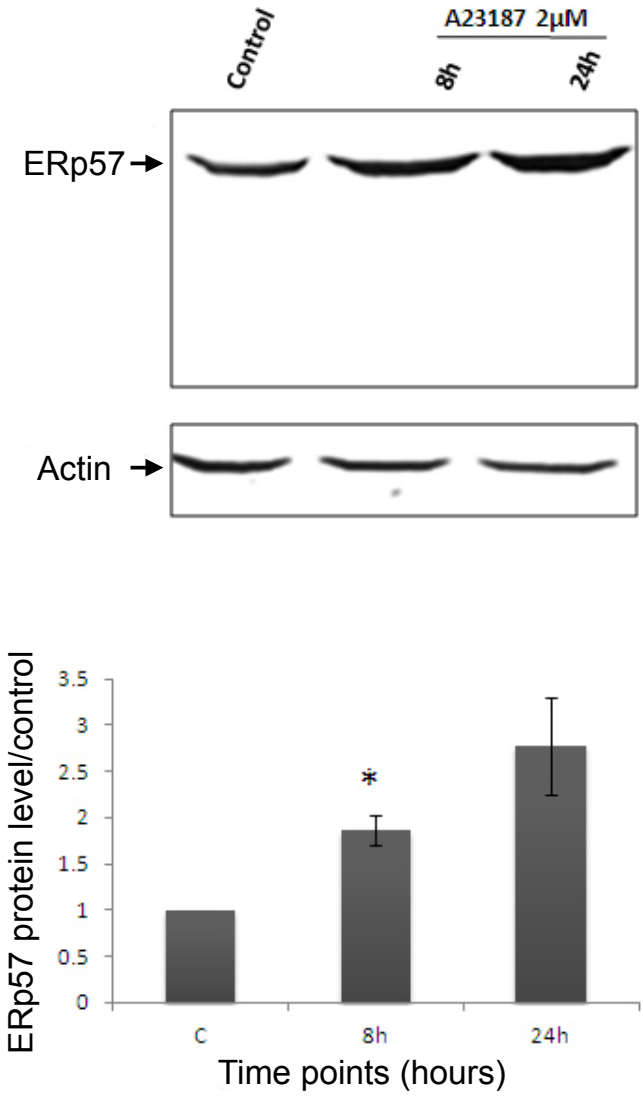

Figure 2.3.8: **Induction of ERp57 protein levels in RTS11 cell line following treatment with A23187.**

Western blot analysis of ERp57 protein levels after *in vitro* induction of ER stress with 2 mM of calcium ionophore A23187 during 24 h time course. Densitometry analysis (lower panel) was conducted using image J software. Results are representative for three individual experiments. Statistical analysis was performed using one-way ANOVA and Tukey's post hoc test. Significant differences between the control and treatment groups is represented by three separate experiments.

2.4 Discussion

2.4.1 Rainbow trout ERp57 gene and protein

Phylogenetic analysis and Southern blotting suggested that two copies of the ERp57 gene existed in the rainbow trout genome. Many rainbow trout genes are duplicated due to an ancestral tetraploidization [113] and therefore it could be suggested that ERp57 is found in two different loci. The lack of a signal peptide and the proximal 20 aa of the conserved domain a' in trout EZ774749 might suggest a possible second gene for ERp57 in trout that has diverged to perform a different function outside of the ER. The trout EZ774749 sequence does however contain a KSKKK motif which might be a much stronger nuclear localization signal than the two lysines found in the ERp57 sequence we cloned. It could be speculated that the duplicated trout ERp57 genes might have diverged in function, with one encoding a protein which primarily performs ER functions, while the other primarily focuses on nuclear functions. This hypothesis is supported by the atlantic salmon sequences as the BT058988 and BT044896, which cluster together in the phylogenetic tree and include a putative nuclear localization signal, while the second atlantic salmon cluster of NM001168519 and BT046042, contain sequences with only two lysines at their C termini. Interestingly, the phylogenetic tree shows that the atlantic salmon BT058988/BT044896 pair which have a strong NLS cluster with the trout sequence which has no NLS, while the trout EZ774749 sequence, which has a potentially strong NLS, clusters with the two salmon sequences lacking an NLS. This suggests that the gene duplicates in these two salmonid species have diverged differentially since the species diverged.

The fact that two mRNA sizes were seen in northern blots also supports the presence of more than one gene in rainbow trout. Interestingly, the presence of the 1.8 kbp lower band in the northern blot seems to be stronger in the liver, head kidney and egg tissues, which imply on a differential expression of the second trout gene in trout. Like its mammalian counterpart, the trout b' domain is enriched with conserved lysines and arginines that are known to bind the negatively charged P domain of calnexin and calreticulin. Interestingly, this negatively charged P domain has been identified in calreticulin of rainbow trout [116] reinforcing the conserved function of this domain in the binding of ERp57 to other chaperones. All teleost and amphibian ERp57 proteins, which includes trout, salmon, zebrafish and xenopus, do not seem to have the classical ER retention signal that is present in ERp57 of mammals and chicken. However, it could be that teleost ERp57 is kept in the ER through its strong interaction with calreticulin, for which the trout version does have

an ER retention signal [116] or with other ER resident proteins.

Another explanation might be that trout ERp57 may play roles in another subcellular location following its secretion from the ER, as it has been shown that mammalian ERp57, despite having an ER retention signal, can exit the ER and localize either to, the nucleus, the cell surface or the extracellular environment [102]. Nuclear localization of ERp57 has been characterized in mammals, chicken, frogs and zebrafish all of which have a C terminal lysine rich nuclear localization sequence. Interestingly, this nuclear localization sequence only contains two lysine residues in trout and salmon, making them different from zebrafish. It could be hypothesized either that ERp57 in trout and salmon have a nonclassical nuclear localization signal or that the protein can be transported to the nucleus by another carrier. It could also imply that the function of both trout and salmon ERp57 in the nucleus has been lost, and that they represent an ancestral version of the protein that has not yet acquired a nuclear function. The polyclonal antibody used here would detect both putative ER resident and nuclear localized forms of ERp57 however. This suggests that both forms are similarly regulated and perhaps that one form is stimulated while the other is not. Development of monoclonal antibodies to both protein isoforms will be needed to dissect potential differential regulation.

2.4.2 Induction of ERp57 expression in rainbow tissues and RTS11 cell line

Tissue distribution of ERp57 was studied by northern blotting hybridization showing a 2 kb mRNA is expressed in all tissue studied, similar to the mRNA size of human ERp57 [114]. In order to investigate the correlation between the level of expression of ERp57 and immune stimulus in rainbow trout, fish were injected with PHA, an agent commonly used for the stimulation of cell division in lymphocytes. Trout ERp57 levels in both gill and spleen were shown to be elevated after 48 and 96 h respectively (Figure 2.3.5). The upregulation after 96 h in the heart might be due to the presence of activated circulating lymphocytes that might express the gene rather than expression by the heart tissue itself. Interestingly, it has been shown that surface expression of human and trout calreticulin in PBL is elevated after PHA stimulation [117, 118]. Thus if ERp57 is responsible for the trafficking of calreticulin to the cell surface [119], that might cause a reduction in the protein level within the ER that is compensated for by enhanced ERp57 transcription. *In vitro* stimulation of RTS11 culture with poly I:C, a synthetic double stranded RNA which

induces expression of antiviral proteins and a transient antiviral state [117, 120, 121], was shown to upregulate ERp57 expression levels in a time dependent manner. Previously poly I:C was shown to increase the induction of MH I pathway genes such as β2m [79] and enhance the expression of Tapasin [122], the peptide transporter TAP1 and the proteasome component LMP7 [122] in ASK (atlantic salmon kidney) cells. Importantly, a similar induction was found in response to infectious salmon anemia virus (ISAV) and *in vivo* during acute infection with infectious hematapoeitic necrosis virus (IHNV) in rainbow trout [99]. Future studies will determine if ERp57 can be upregulated in virally infected cells as part of the host interferon response.

Upon short term induction of ER stress using the calcium ionophore A23187, trout ERp57 protein levels were increased both in RTS11 and PBL, similar to the upregulation of human ERp57 [123] and other ER resident chaperones such as calreticulin [124] and Bip [125] during ER stress. The PCR primers used in the induction studies reported here are specific for the sequence we isolated which lacks the nuclear localization signal, thus may encode the ER specific version of the ERp57 protein. These results imply that trout ERp57 may have a conserved function in the ER and during the unfolding protein response despite the lack of a conventional ER retention signal. Interestingly, it has been shown that trout calreticulin expression levels are not induced either by poly I:C or calcium ionophore [117] which might suggest that ERp57 has a more conserved role in the ER than calreticulin in fish. It may be possible that ERp57 upregulation can enhance the recruitment of "free" calreticulin to the MHC class I complex through their known interaction [120].

2.5 Summary

This work demonstrates that trout ERp57 is a conserved protein that shares high amino acid sequence identity and possible similar functions with its mammalian homolog. Nevertheless, there are some differences in structure, such as the ER retention signal and the nuclear localization signal that do not appear to be conserved in the trout sequence and therefore might point out to new mechanism of action and translocation from the ER. This work suggests that there are two gene copies of ERp57 in trout that might have diverged to perform various functions in different cellular compartments. The putative role of ERp57 during ER stresses and in the assembly of the MH class I protein loading complex might help to understand the pathology of viral diseases in fish. Our findings that trout ERp57 expression levels are upregulated both upon ER stress and poly I:C treatment, might sug-

gest for the first time in teleosts, a possible role of ERp57 during viral induced ER stress and interferon responses. Future work will determine ERp57 subcellular localization and its interactions with other molecular chaperones as part of the peptide loading complex of MH class I.

Chapter 3

The cloning and characterization of trout calnexin gene

3.1 Introduction

Calnexin (IP90/P88) is an integral membrane protein of the endoplasmic reticulum that binds to monoglycosylated oligosaccharides. It was first identified as a protein that associates with partly folded MHC class I molecules, T cell receptors and membrane immunoglobulins [28, 126, 29]. Together with calreticulin, its soluble homologue, it participates in the calnexin/calreticulin cycle responsible for folding and quality control of newly synthesized glycoproteins before their export from the ER [127, 128, 22]. Calnexin has also been shown to play a role in ER calcium regulation [26, 129], phagocytosis [130] and cell sensitivity to apoptosis [131, 132, 133, 134].

Two distinct luminal domains of calnexin were revealed by crystallization [135]: the globular domain that contains the lectin binding site and an extended arm domain, containing two tandem proline rich motifs, which binds to ERp57 [136]. The C terminal domain is highly acidic with a protein kinase-dependent phosphorylation site that plays a possible role regulating the chaperone function [137, 138].

One well investigated function of calnexin is stabilizing both MHC class I and class II molecules [139, 140]. In humans, newly translated MHC class I heavy chain associates with calnexin rapidly, but it is released and replaced by calreticulin upon assembly of heavy chain with β2m. However, in murine cells either calnexin or calreticulin may associate with β2m-heavy chain dimers. Interestingly, experiments assessing the function of

calnexin in the biogenesis of class I molecules showed contradicting results. Co-expression of calnexin or calreticulin with MHC class I heavy chain in *Drosophila melanogaster* cells demonstrated five fold enhanced assembly with the $\beta 2$ microglobulin, while treatment with ER glucosidase I and II, inhibitors of monoglycosylated oligosaccharide formation, reduced the assembly and surface expression of MHC class I [23]. However, other experiments using calnexin human deficient cell lines showed no effect at all on class I assembly, transport or peptide loading [30, 141]. These experimental discrepancies may be either explained by the different model systems used or by the redundancy of molecular chaperones that can functionally replace calnexin in the ER such as calreticulin [142].

Co-immunoprecipitation studies have also demonstrated that calnexin can function as chaperone for some viral glycoproteins such as the G protein of vesicular stomatitis virus (VSV) and more recently the S glycoprotein of severe acute respiratory syndrome coronavirus (SARS coronavirus) by assisting in their folding and full maturation [143, 144]. Calnexin homologues have been identified in many eukaryotes such as plants, yeast, Xenopus [145, 146, 147], several fish species [148], *Danio rerio* (NM_213448.1), *Takifugu rubripes* (XM_003978229 and XM_003978694) and remarkably in *Dictyostelium* [130] which implies possible conserved functions for this molecule. To date, calnexin's role in teleost fish has only been investigated in channel catfish where it was shown to associate both with the non-glycosylated MHC class II alpha chain and the glycosylated β chain [148]. However, no studies have been carried out to assess its role during MHC class I folding, MHC class I receptor assembly in the ER or its possible association with viral glycoproteins mainly due to the lack of available antibodies. In rainbow trout, besides calnexin, most of the genes encoding molecular chaperones involved in this pathway have been fully characterized such as tapasin [86], calreticulin [116] and recently ERp57 [149]. In this work two unique cDNA clones for calnexin from trout PBL are reported and their regulation under ER stress induced conditions is described.

3.2 Materials and methods

3.2.1 Fish

Rainbow trout were obtained from Silver Creek Aquaculture (Erin, ON) and kept at 13°C in 200 litre fresh-water flow-through tanks at the University of Waterloo under 12:12 h light-dark cycle. Fish were fed 5 days/week with commercial dry pellet (5 point floating

trout chow from Martin Mills, Elmira, ON). Animals were kept using CACC guidelines under a permit from the University of Waterloo animal care committee. Adult fish (800 $\sim$ grams) were anesthetized in 1 mL/liter of 2-phenoxyethanol (Sigma Aldrich St. Louis, MO). Blood was drawn from the caudal sinus as previously described [149] and tissues samples were collected in RNA later (2.5 mM Na citrate, 5.3 M $(NH_4)_2SO_4$, 0.01 M EDTA, pH 5.2).

3.2.2 Rainbow trout cell lines

The rainbow trout cell lines utilized were the monocyte/macrophage cell line, RTS11 [107], and the epithelial-like cell lines: RTL-W1 from a normal liver [108], RTgill-W1 from the gill [109] and RTovarian fluid [TK Vo and Bols]. RTS11 was grown at 20°C in L-15 media with 15% FBS and other cell lines were grown in 10% FBS as described by Kawano et al. [79]. The epithelial cell lines maintained epithelial-like morphology during the course of this study.

RTS11 stimulation with A23187 calcium ionophore and poly I:C

Cultures were seeded to 2×10^6 in a $25\,cm^2$ flasks containing L-15 media supplemented with 150 U/mL of penicillin and 150 mg/mL streptomycin in 15% fetal bovine serum (ThermoFisher Scientific, Nepean, ON). Treatment included either 2 μM of A23187 dissolved in DMSO or 50 μg/mL of polyinosinic-polycytidylic acid (poly I:C) in PBS (Sigma Aldrich, St. Louis, MO). The same volume of vehicle was added to the control.

3.2.3 Cloning of rainbow trout calnexin cDNA

Degenerate primers were designed based on conserved regions identified by alignment of the following sequences from GenBank: *Homo sapiens* [NP_001019820.1], *Mus musculus* [NP_031623.1], *Canis familirais* [NP_001003232.1], *Rattus norvegicus* [NP_742005.1], *Danio rerio* [XP_002665576.1] and *Ictalurus punctatus* [AAQ18011.1]. Total RNA was extracted from 2×10^6 RTS11 cells using Qiagen RNeasy extraction kit according to manufacturer's instructions (Qiagen, Mississauga, ON), followed by single strand cDNA synthesis using a Fermentas RevertAid First Strand cDNA Synthesis Kit with one μg of total RNA (ThermoFisher Scientific, Nepean, ON). PCR reactions of 25 μL included 1x PCR buffer, 200 μM dNTP mix, 2 mM MgCl2, 10 μM each of the forward 5' AARTAYGAYGGNAARTGG

3' and reverse primer 5' TCTTCTTTCCAGTGCAGCAGA 3', 1 μL of RTS11 cDNA and 1U of Taq Polymerase (MP Biomedical, Solon, OH). The reaction parameters were 95°C for 5 min followed by 30 cycles of (95°C 40 sec, 50°C 30 sec, 72°C 2 min) with a final 15 min 72°C using a BioRad DNA Engine thermocycler (BioRad, Mississauga Ontario). An estimated 1200 bp PCR product was purified using a Qiagen gel extraction kit (Mississauga, ON), then ligated into pGEM-T Easy vector, subcloned into XL1-Blue MRF' E. coli competent cells and sequenced at The Centre for Applied Genomics (Toronto, ON, Canada as described in [149].

Generation of full length cDNA

The full 5' end of the calnexin transcript was obtained using RACE adapted from [106] and described in detail in [149]. Briefly, cDNA synthesis used four μg of total RNA derived from peripheral blood leukocytes and gene specific primer (GSP1) 5' CATGTCCTCCACCTCCCA3'. Reverse transcription was performed for 60 min at 42°C, 5 min at 45°C, 5 min at 50°C and 10 min at 70°C followed by addition of RNAseH (Invitrogen, Carlsbad, CA), dATP and terminal deoxynucleotidyl transferase (Invitrogen, Carlsbad, CA) as described [149]. The PCR reaction was performed using 10 μM gene specific primer sense primer 2 (GSP2) 5' CGGGCGCTTTGTAGGTCACTT 3' combined with the RACE primer 5' CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCTTTTTTTTTTTTT 3' and 2 μLof template obtained from the 5'cDNA pool.

The PCR reaction for the first cycle was performed under the following conditions: 95°C for 5 min, 48°C for 2 min and 72°C for 15min with additional 30 cycles of 94°C for 15 sec, 52°C for 30 sec and 72°C for 3min. Sequence alignment of the 3' UTR region of both *Ictalurus punctatus* NM_001200180 and *Danio Rerio* NM_213448 revealed a conserved region of 18 nucleotides which facilitated the design for a PCR primer to amplify this end of the cDNA. The primers that were used in this PCR reaction were as follows: 5' ATGGAGTTGAATGTGAGGTGTG 3' and anti-sense primer 5' AAGTCCATCAGTCCTTTCT 3'. The PCR reaction to amplify the 3' UTR was performed under the following conditions: 95°C for 5 min, 53°C for 30 sec and 72°C for 4 min followed by with additional 30 cycles of 94°C for 3 min, 53°C for 30 sec and 72°C for 3 min.

3.2.4 Southern blot analysis

Genomic DNA obtained from rainbow trout peripheral blood leukocytes was extracted using a Wizard Genomic DNA purification kit (Promega, Madison, USA). Briefly, ten μg of genomic DNA was completely digested for 4 hours at 37°C using Fast digest enzymes *Eco*RV, *Hin*dIII, *Kpn*I and *Pst*I and *Bam*HI (ThermoFisher Scientific, Nepean, ON), separated on a 1% agarose gel and transferred to a positively charged membrane (Roche, Manheim, Germany), followed by a UV cross linking. Membranes were washed and hybridized with DIG labelled probes as previously described [149]. Two distinct probes were generated either to the proximal or distal end of calnexin. The primers used to generate the 477 bp proximal probe was as follows: forward 5' AGGAGGACATTGATGAGGATATTGC 3' and reverse 5' GACCCACCTTTACACTCGGTGGTGAACCCAG 3'. For the distal 540 bp probe primers were as follows: forward 5' TGGATGATCAGCCAGAGTACA 3' and rev 5' TGCAGAAGGAAGACAATGATGAGGA 3'. The bands were detected using anti-DIG antibody conjugated to alkaline phosphatase and CDP star (Roche, Mannheim, Germany).

3.2.5 Northern blotting analysis

RNA was isolated from both rainbow trout tissues and the RTS11 cell line using Trizol according to manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Briefly eight μg of RNA from tissues and six μg RNA obtained from RTS11 cell were separated on a 1% agarose-formaldehyde gel and transferred to a positively charged membrane (Roche, Manheim, Germany), followed by UV crosslinking. The membrane was washed, hybridized and detected as previously described [149]. The primers used to generate the 728 bp probe were as follows: forward 5' TGGTGGTGAACCCAGAC 3' and reverse 5' CCAGGGACGC TCGTCTGCTG 3'.

3.2.6 Phylogenetic tree construction

Calnexin nucleotide alignment was generated using Muscle [150] and was used to construct a phylogenetic tree using the neighbour-joining method [151] with the Jukes and Cantor correction [152] for 1,000 bootstrap replications. Numbers above the tree lines indicate the percent bootstrap confidence values. Nucleotide sequences were obtained from the GenBank database and are listed in Table 3.1.

Species	Accession number
H. sapiens	NM_001024649.1
P. troglodytes	XM_003310996.2
B. taurus	NM_001105612.1
C. lupus	NM_001003232.1
M. musculus	NM_007597.3
R. norvegicus	NM_172008.2
G. gallus	NM_001030620.1
D. rerio	NM_213448.1
C. elegans	NM_066775.3
O. sativa	NM_001059227.1
A. thaliana	NM_125573.3
X. laevis	NM_001086857
X. tropicalis	NM_001005668
D. melanogasterr	M_170407.3
T. rubripes	XM_003978229
T. rubripes	XM_003978694
O. latipes	XM_004084923
O. latipes	XM_004065645
O. niloticus	XM_003447644
I. punctatus	NM_001200180.1

Table 3.1: Calnexin nucleotide sequences obtained from the GeneBank database.

3.2.7 Sequence analysis

SignalP 3.0 server was used to identify the signal peptide [153], while TMHMM Server 2.0 was used to identify transmembrane regions [154], NetPhos 2.0 Server was used to identify possible phosphorylation sites [155], palmitoylation sites were predicted by CSS-Palm 3.0 [156] and both InterProScan (EBI) [157] and the NCBI conserved domain (CDD) platform [158] were used to identify protein conserved regions.

3.2.8 Western blot analysis

Cell pellets were lysed in 1% NP-40 lysis buffer containing 150 mM NaCl and 50 mM Tris [pH 8.0] supplemented with 1X protease inhibitor cocktail (Roche, Manheim, Germany). Thirty μg of protein samples were separated on a 10% acrylamide gel and transferred to nitrocellulose membranes (Bio-Rad, Mississauga, ON) overnight. Membranes were blocked with 5% skim milk in TBS-T (136 mM NaCl, 2 mM KCl, 2.4 mM Tris, 0.05% Tween 20)

and probed with 1:500 rabbit polyclonal antibody for human calnexin GTX101676 (GeneTex Irvine, CA) followed by 1:30000 goat anti-rabbit alkaline phosphatase (Sigma Aldrich St. Louis, MO). Bands were detected using NBT/BCIP (Roche, Manheim, Germany) according to manufacturer's instructions.

3.2.9 Isolation of peripheral blood leukocytes

Trout peripheral blood was centrifuged at $400 \times g$ for 10 min, a buffy coat was obtained and diluted 1:10 with L-15 media (Sigma Aldrich St. Louis, MO). Diluted fractions were layered on the top of 3 mL histopaque 1077 (Sigma-Aldrich, St. Louis, MO) and centrifuged for 30 min at $400 \times g$. White blood cells were collected, centrifuged and washed once with 1X PBS for 5 minutes. Pellets were stored in -80°C until later use.

3.2.10 Immunofluorescence

RTS11 cells were seeded to 2×10^5 cells per well in 4 chamber slides containing 2% FBS 150 U/mL of penicillin and 150 mg/mL streptomycin and grown overnight. All steps were conducted at 20°C. Cells were washed once with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4; pH 7.4) and fixed for 15 min with 4% paraformaldehyde (Thermo Fisher Scientific, Nepean, ON) followed by two washes with PBS. Cells were permeabilized by incubation for 10 min with PBS containing 0.25% Triton X-100 (PBST), followed by 3 washes with PBS of 5min each. Cells were incubated with 1% BSA in PBST containing 0.1% (v/v) of Triton X-100 for 30 min and then probed with 1:100 of polyclonal rabbit anti-human calnexin (GTX101676) in 1% BSA for 1 h. After three washes for 5 min each in PBS, cells were incubated for 1 h with 1:3000 of goat anti-rabbit Alexa Fluor 488 (Invitrogen, Carlsbad, CA) in 1% BSA followed by 3 washes with PBST of 5 min each. Cells were dried for 5 min at room temperature, mounted with Fluoroshield containing DAPI (Sigma Aldrich St. Louis, MO), coverslips were attached and the slides were stored at 4°C. Slides were examined by laser scanning confocal microscopy using a Zeiss Axiovert 200 microscope and ZEN 2009 software (Carl Zeiss, Mississauga, ON).

3.3 Results

3.3.1 Sequence analysis

An estimated 1230 bp amplicon was isolated from RTS11 cDNA which showed 83% nucleotide identity with *Ictalurus punctatus* calnexin (NM_001200180.1). In order to obtain the 5' end and 3' end of the trout calnexin cDNA, PCR primers were used for 5' RACE and for a conserved region in the 3' UTR using a PBL cDNA template. The corresponding PCR fragments were 380 bp and 320 bp respectively. PCR amplification of the full open reading frame of rainbow trout calnexin was then conducted using 5' and 3' gene specific primers using PBL cDNA template. Interestingly, two unique cDNA clones similar in length were obtained.

The first calnexin cDNA clone contains 1812 nt in its coding region and 94 nt of 3' UTR corresponding to putative 603 aa protein of 68 kDa. The second cDNA clone contains slightly longer sequence of 1851 bp with additional 23 bp of 3' UTR (117 bp total) which encodes a 616 aa protein with predicted molecular weight of 70 kDa. Both cDNAs showed 73% and 79% nucleotide identity with *Homo sapiens* and *Ictalurus punctatus* calnexin sequences, respectively with 72% and 75% sequence identity to their encoded proteins. The main characteristic of the rainbow trout calnexin proteins are illustrated in Figure 3.3.2. The N terminal domain includes a 24 aa signal peptide, followed by a luminal domain that contains a globular domain and an extended arm domain with a central segment, the P domain. This segment contains two proline motifs: M1 and M2 repeated in tandem four times each with the consensus sequences of M1: I-DP(D/E)A-KPEDWD(D/E) and M2: G-W-P-IN-P-Y. Moreover, there are three regions that have high sequence identity with human calnexin that are named box a, b and c. The box a segment is located upstream to calreticulin domain 1, whereas box b and c flank the repeated motifs. There is also a transmembrane domain of 21 aa that was predicted by TMHMM Server.

Interestingly, there are two pairs of cysteines predicted to be palmitoylated by CSS-Palm 3.0 immediately after the transmembrane domain. These cysteines are found to be conserved across many species and when palmitoylated were shown to be critical for the association of calnexin with the ribosome translocon complex. This helps calnexin to capture the N-linked glycans as they exit from the translocon [159]. Another shared feature with mammalian calnexin is the presence of an acidic cytoplasmic tail enriched with glutamic acid and ER retention signal KQEDDL, which appears only in one of the clones and interestingly does not appear in the channel catfish sequence [148]. There are

five phosphorylation sites predicted by NetPhos 2.0 Server for both trout clones, however a unique serine phosphorylation site downstream of the ER retention signal known to regulate calnexin activation [160] exists only the clone with the ER retention motif.

Figure 3.3.1: **Sequence alignment of trout calnexin with known nucleotide sequences.** Calnexin predicted nucleotide sequences were aligned with other known vertebrate sequences obtained from the GenBank (see methods for specific accession numbers). Numbers on the right indicate amino acid positions. The conserved domains are labelled in bold above the sequences. The palmitoylation site in the juxtamembranal region is underlined. Both the putative ER retention signals and the serine phosphorylation site are labelled in bold and underlined.

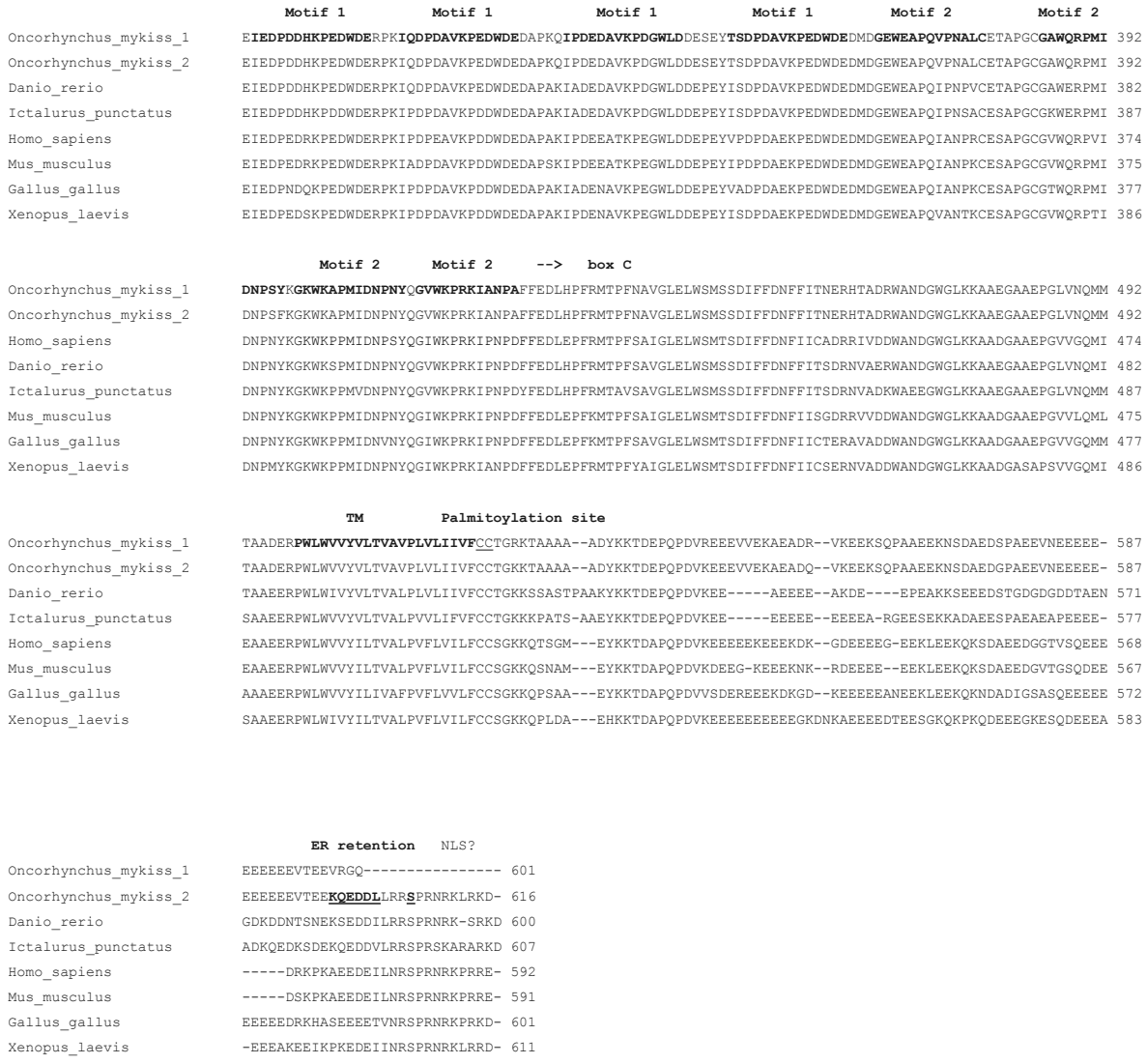

Figure 3.3.2: **Sequence alignment of trout calnexin with known nucleotide sequences.** Calnexin predicted nucleotide sequences were aligned with other known vertebrate sequences obtained from the GenBank (see methods for specific accession numbers). Numbers on the right indicate amino acid positions. The conserved domains are labelled in bold above the sequences. The palmitoylation site in the juxtamembranal region is underlined. Both the putative ER retention signals and the serine phosphorylation site are labelled in bold and underlined.

3.3.2 Phylogenetic analysis

In order to further examine the evolutionary relationship between the unique trout clones and other calnexin nucleotide sequences, a phylogenetic tree were constructed (Figure 3.3.3). As expected, strong distinct clusters can be seen for the tetrapod, teleost fish and plant sequences. Both trout sequences clustered together with a short genetic distance suggesting a recent gene duplication event, which is common in salmonids [161]. Interestingly a more ancient duplication event also seems to have occurred in teleost fish, as both pufferfish, and rice fish showed a second sequence which grouped with zebrafish calmegin outside the main cluster of fish, frog and mammalian calnexin sequences, but still within a vertebrate cluster. This suggests a very early duplication event to produce this testis-specific ER chaperone homologous to calnexin.

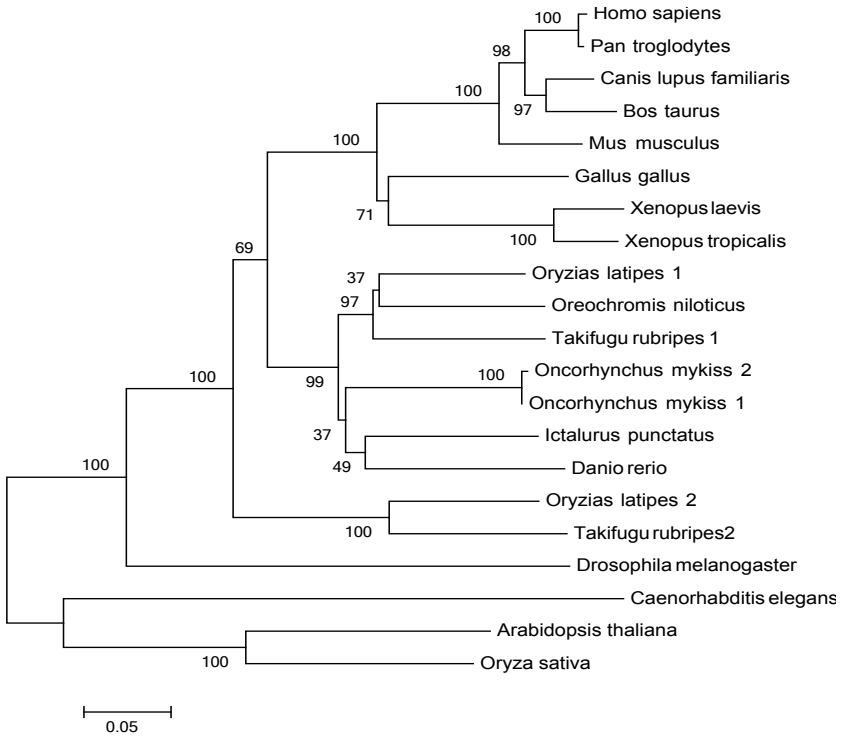

Figure 3.3.3: **Phylogenetic analysis of known calreticulin nucleotide sequences.**
A phylogenetic tree constructed using known complete coding sequences of calnexin obtained from the GeneBank data base. The sequences were aligned using Muscle program and phylogenetic tree was constructed by Mega4 using neighbour joining method with the Jukes and Cantor correction. Numbers indicate on bootstrap values determined through 1000 replications. The bar at the bottom indicates Neis genetic distance.

3.3.3 Trout calnexin gene copy number

To further examine the possibility that the calnexin gene might be duplicated in trout, two separate probes were designed to proximal and distal segments of the calnexin gene. When the proximal probe was used, between three to four bands were observed in each digest (Figure 3.3.4A). The distal probe showed similar results showing two dark bands and either one to two faint bands (Figure 3.3.4B). These results suggest that the calnexin gene is present in at least two copies in trout, unlike its soluble paralog calreticulin which appears in rainbow trout as single copy gene [116].

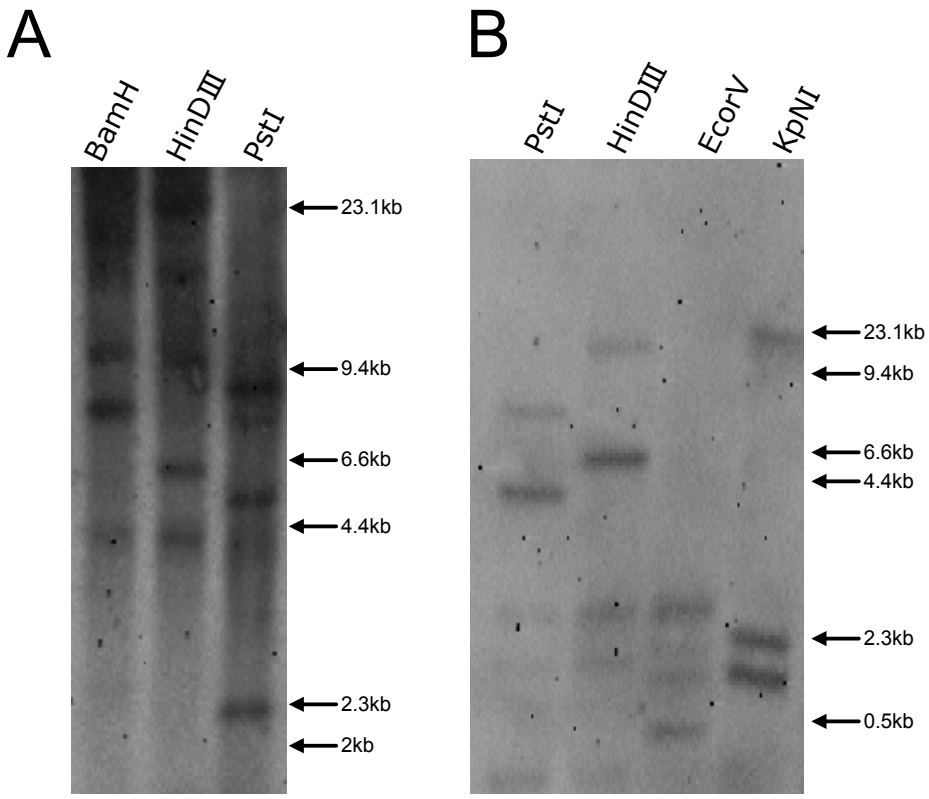

Figure 3.3.4: **Southern blot analysis of trout calnexin.**
Southern blots of rainbow trout calnexin gene. Ten micrograms of genomic DNA was isolated from rainbow trout peripheral blood leukocytes and was digested using *Eco*RV, *Hin*dIII, *Kp*nI and *Pst*I and *Bam*H. Digests were hybridized with either a DIG labelled cDNA probe spanning the proximal domain of trout calnexin (A) or with a probe spanning the distal domain of trout calnexin (B). The position of the size marker (λ/*Hin*dIII digest) is shown in kilobase on the right margin.

3.3.4 Calnexin tissue expression

Northern blotting shows wide tissue distribution of the calnexin mRNA in trout tissues with the most prominent message being an estimated 3 kbp transcript band but with a faint lower band detected in spleen, head kidney and PBLs (Figure 3.3.5). This lower band was strongly expressed in RTS11 (Figure 3.3.6).

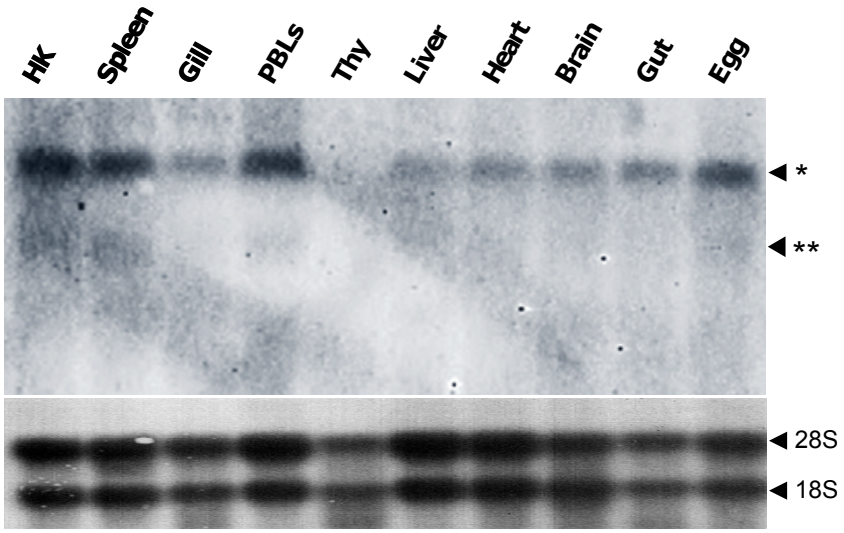

Figure 3.3.5: **Calnexin transcripts distribution in trout tissues under non stimulating conditions.**
Eight μg of RNA from healthy trout tissues was separated on a 1% agarose-formaldehyde gel. Calnexin transcript was detected as a prominent band around 3kb and indicated by a single asterisk. An additional low expressed transcript was detected in spleen, head kidney and PBLs and indicated by a double asterisk. The size of the 28S and 18S ribosomal RNA bands is shown in the lower panel.

In order to determine if both bands were similarly regulated, RTS11 cells were treated with A23187, which is known to induce ER stress and upregulate other molecular chaperones *in vitro* such as ERp57 [149]. Importantly, the lower calnexin transcript was upregulated up to 2.6 fold in 24 h followed by a decreased expression at 36 h. Interestingly, the upper band expression remained relatively constant Figure 3.3.6. This result points out to the possibility that trout may have two unique transcripts responding differentially to ER induced stress due to unique modes of regulation.

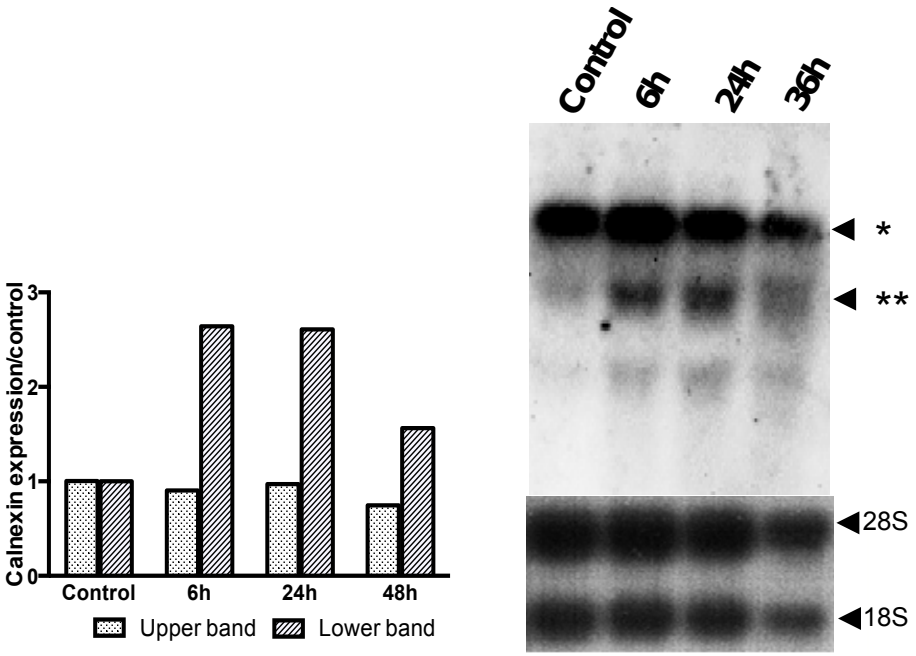

Figure 3.3.6: **Upregulation of calnexin mRNA in RTS11 cells following treatment with calcium ionophore A23187.**

Northern blot analysis showing calnexin mRNA levels upon stimulation with 2 μM of the calcium ionophore A23187 for 36h. Densitometry analysis on the left panel was conducted using image J software and is expressed as the ratio between calnexin and ribosomal 28S bands. The detection of the higher calnexin transcript band is indicated by a single asterisk, whereas the expression of the lower and induced transcript band is indicated by a double asterisk. This result represents a single experiment.

3.3.5 Calnexin protein levels under normal and induced ER stress conditions

In order to further explore the possible role of calnexin during ER stress, an anti-human calnexin antibody (GTX101676) was used. This antibody was generated against the recombinant P domain of human calnexin, which shares 81% identity with the trout calnexin sequence. As opposed to its 70 kDa predicted molecular weight, trout calnexin protein was detected as a 120 kDa band in RTS11, RTgill, RTgut, RTovarian fluid and RTliver (Figure 3.3.7A and B). Interestingly, a lower fainter band around 100 kDa was also detected in all cell lines tested. A doublet band around 55 kDa was observed in RT liver, probably due to antibody binding to calreticulin, known to migrate as a doublet band in trout [117]. In channel catfish calnexin is detected as a single band around 100 kDa compared to its 69 kDa molecular weight [148]. This discrepancy is common in many species and might be explained by the abundance of acidic amino acids which interferes with SDS binding and reduce the mobility on SDS-PAGE, and post-translational modifications. Importantly,

induction of RTS11 with A23187 in a set of three repeats showed detection of the lower band in the treated cells but not in the control (Figure 3.3.7C). RTS11 treated with poly I:C didnt show any induction of the lower band (data not shown) which suggests that the expression of the smaller version of calnexin is modified specifically under ER stress conditions.

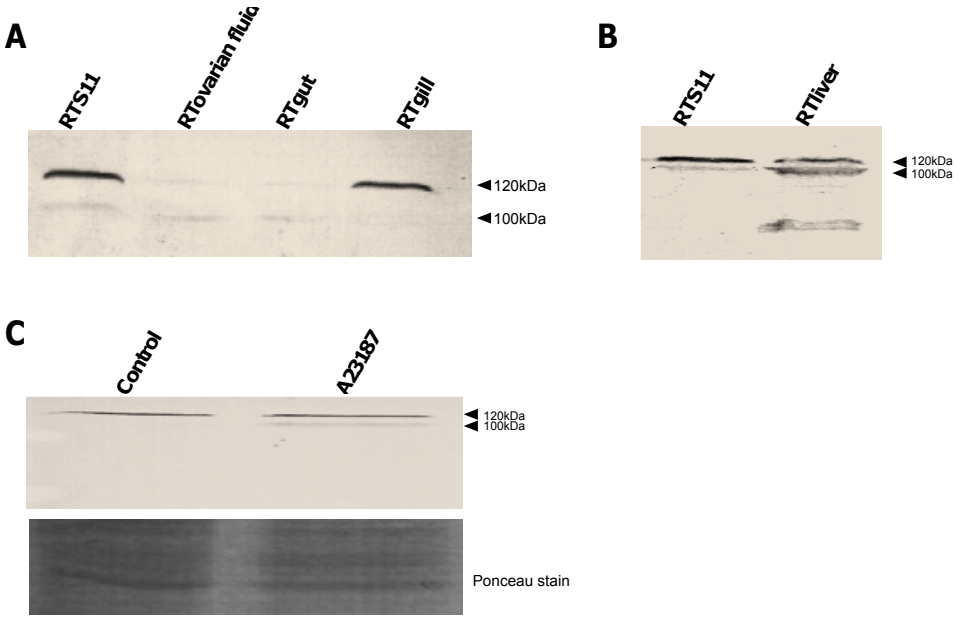

Figure 3.3.7: **Calnexin protein distribution in rainbow trout cell lines under non stimulating conditions and during *in vitro* stimulation with A23187.**
Western blot analysis of calnexin protein levels in trout cell lines using anti-human calnexin polyclonal calnexin antibody. Unstimulated cell lysates were separated on a 10% (A) or 12% SDS–PAGE gel (B) and during 24 h treatment with calcium Ionophore A23187 (C). This experiment is representative of three replicate set. The position of the size marker is shown on the right margin.

3.3.6 Calnexin localization in RTS11 cells

In order to further validate the specificity of the GTX101676 antibody, RTS11 were stained with anti-calnexin antibody (GTX101676) and examined under the confocal microscope (Figure 3.3.8). The staining revealed a meshwork structure within the cells with additional staining observed surrounding the nucleus. A similar and stronger staining pattern was observed for other ER resident proteins, trout ERp57 and calreticulin, the latter of which contains a putative ER retention signal [116]. These results suggest that trout calnexin might reside in the ER. However, additional experiments are required to validate the cellular localization of calnexin in trout.

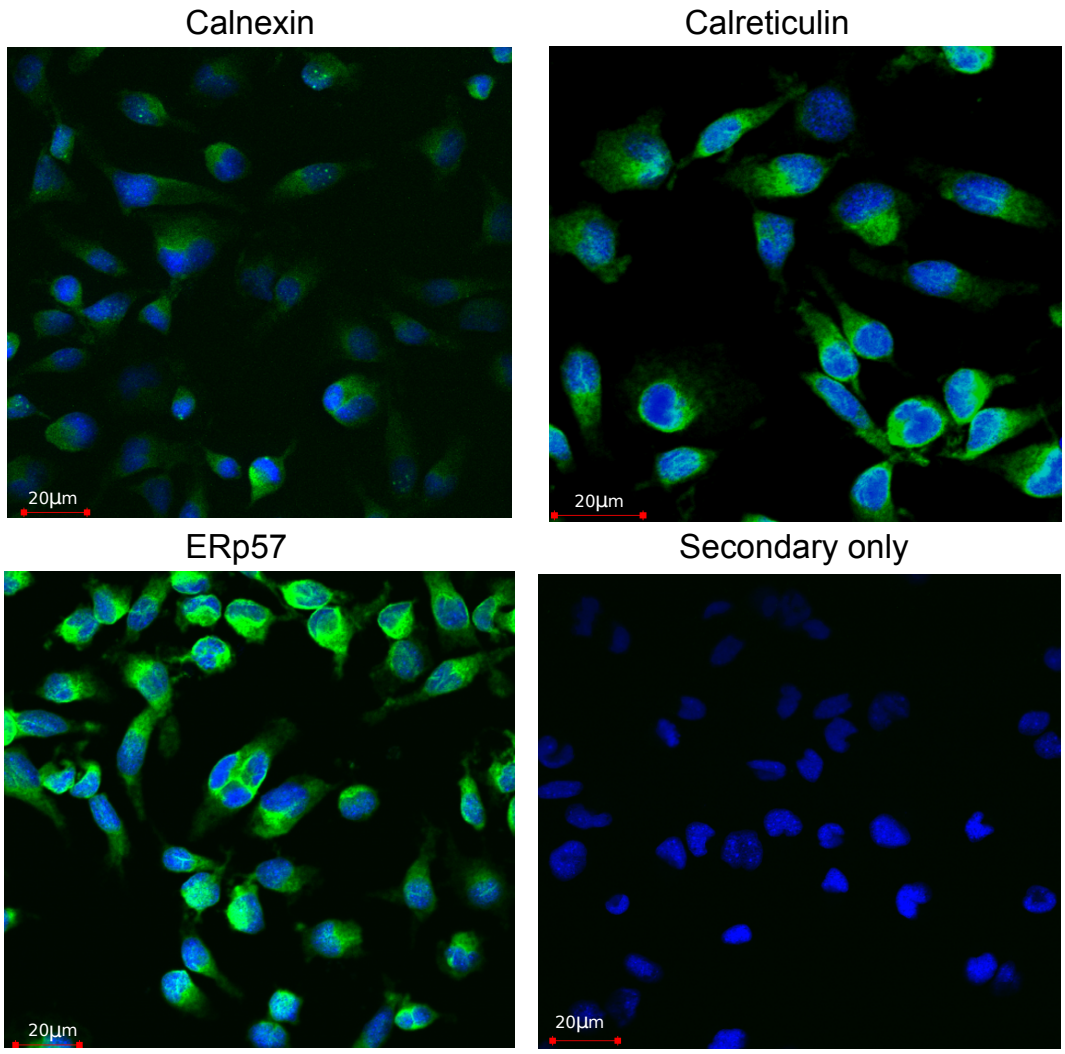

Figure 3.3.8: **Localization of calnexin, ERp57 and calreticulin in RTS11 cells.** Immunofluorescence detection of calnexin, ERp57 and calreticulin in RTS11 using anti human calnexin GTX10167, and antibodies generated against trout ERp57 and calreticulin proteins. Alexa Fluor 488 was used as anti rabbit secondary antibody. Fluoroshield containing DAPI was used for nuclear staining. The negative control was stained with secondary antibody alone.

3.4 Discussion

In this study, rainbow trout calnexin was characterized and its expression was investigated to examine the regulation of a possible peptide loading complex in fish using rainbow as a model organism. Two unique cDNA clones encoding trout calnexin which share 99% sequence identity in their coding region were identified. Intriguingly, only one cDNA version contains a unique C terminal with putative ER retention signal and conserved serine phosphorylation site which activates calnexin when protein misfolding occurs and is important for the recruitment of calnexin to ribosome translocons [162, 138, 159]. Sequence alignment with the human calnexin protein reveals 72% identity and implies structural and functional conservation of calnexin as an ER lectin chaperone, calcium binding protein and as a protein indirectly assisting in oxidative folding.

The luminal domain of calnexin contains the two conserved domains: the globular and the arm domain. The lectin binding site was found in a pocket on the surface of the globular domain [135] whereas the tip of the arm domain contains the docking site that recruits ERp57 [136] and therefore helps to support oxidative folding in the ER. The P domain in the central arm segment contains two conserved motifs repeated four times each which contain low capacity and high affinity calcium binding sites, while the C terminal domain is highly enriched with acidic amino acids shown to be high capacity low affinity calcium binding sites [163]. All of these features are evident in the trout calnexin sequences, suggesting that many of its roles are conserved in trout, however the differences in the C terminal domains between the calnexin cDNA sequences might suggest a unique mode of regulation and perhaps differential subcellular localization.

A phylogenetic tree constructed with calnexin nucleotide coding sequences indicates on a possible recent gene duplication event for trout similar to that seen for trout ERp57 [149], but suggesting that both are *bone fide* calnexin homologues. A duplication of calnexin like sequences of pufferfish and rice fish obtained from different loci may be more ancient as these sequences cluster outside all of the vertebrate calnexin sequences. Sequence alignment of their corresponding proteins reveals sequence identity of 73% and 84% respectively to zebrafish calmegin, a calnexin homologue in the testis that is expressed exclusively during spermatogenesis both in mouse and human and was suggested to bind sperm surface proteins important for egg-sperm interaction [164, 165, 166].

In order to investigate the regulation of calnexin during ER stress, cells were treated with calcium ionophore A23187 which has been previously shown to upregulate calnexin

transcript expression by up to 2.5 fold in *Saccharomyces pombe* and appeared as a single band in a northern blot [167]. Interestingly, in trout two possible transcript bands for calnexin were observed that are expressed only in immune system organs. Surprisingly, ER stress induction in RTS11 using A23187 showed that the smaller, less abundant transcript band was induced in contrast to the main transcript which remained unchanged. Similar results under the same stimulating conditions were obtained at the protein level showing the induction of a less abundant 100 kDa band with no change in expression of the higher molecular weight protein band. These results support the presence of two trout genes for calnexin that are regulated differently under ER stress conditions and perhaps performing unique biological functions. Importantly, according to their predicted cDNA sequences their difference in size might be more due to post translational modification rather than simply amino acid differences.

In contrast to what was seen here with trout calnexin, trout calreticulin was identified as a single copy gene in trout and failed to upregulate under several ER stress conditions [117]. This suggests that fish calnexin may perform functions during ER stress that calreticulin cannot. Under normal conditions, trout calreticulin expression was found to be highest in the liver [117] while trout calnexin seems to be more abundant in immune system organs. Interestingly, while calnexin deficient mice display impaired motor function they are not embryonic lethal [168] while calreticulin deficient mice exhibit an embryonic lethal phenotype due to a defect in heart development [169]. In addition, it has been recently demonstrated that both genes are important for generation of neuromasts during lateral line development in zebrafish using calnexin and calreticulin knockout morpholinos, however the calnexin knockout had a more profound effect on this development [170]. These results suggest that both in mammals and in fish, calreticulin and calnexin play a role during embryonic development, which suggest these two proteins may have distinctive roles outside of their chaperone function. Monitoring the expression of these two calnexin genes during stages of embryogenesis in rainbow trout could shed a light on their possible unique functions during development.

The immunocytochemistry of calnexin in RTS11 suggests that calnexin is localized to the ER and possibly resides close to the nucleus, as in some cells it showed staining near the nucleus. Interestingly, calnexin has been found as the major calcium binding protein in rat hepatic nuclear membranes although appeared as a minor member of the total nuclear protein [171]. In addition, immunostaining of calnexin in rat epithelial cells has demonstrated a reticular network structure and nuclear envelope staining [26] supporting

our findings. It may be that the trout calnexin without the ER retention signal can escape the ER and perhaps be localized in the nuclear periphery where it can regulate calcium levels in this region. However, further experimentation with monoclonal antibodies to each specific form is required to see which form(s) is localized to the nuclear region.

The role of calnexin as a chaperone has been widely investigated in mammals, however some research conducted in yeast and the microorganism *Dictyostelium* shed a light on the other important cellular functions of calnexin such as during apoptosis and phagocytosis. The first report linking calnexin to apoptosis was conducted in an *S. pombe* yeast two hybrid system demonstrating that calnexin is required for cell death mediated by the interaction of its cytosolic tail with the pro-apoptotic protein BAK [172]. Later studies in mammals using calnexin-deficient cells further demonstrated its possible role in mediating apoptosis as these cells showed resistance to ER stress induced apoptosis [133, 173]. Another surprising function for calnexin came from a study with *Dictyostelium*, the only microorganism containing both calnexin and calreticulin proteins. This study showed that co-disruption of calnexin and calreticulin results in severely impaired phagocytosis and compromised growth of the phagocytic cups [130], pointing to a possible primary function for calnexin in this organism. This highlights the significance of studying the evolution of this molecule. This study reports the first identification of calnexin sequences in a salmonid species, suggests that this gene is duplicated in the trout genome and shows for the first time the possible differential regulation of the two forms under ER stress stimulating conditions, which is not seen for calreticulin. Further research will focus on its role in the MH class I assembly and its possible interaction with fish viral glycoproteins.

Chapter 4

Tapasin protein distribution in rainbow trout and its regulation during viral mimic and viral infection

4.1 Introduction

Major histocompatibility complex (MHC) class I receptors are expressed on the cell surface of all nucleated cells and present peptides derived from intracellular proteins to CD8+ T lymphocytes. Peptides are generated by the proteasome and delivered into the lumen of the endoplasmic reticulum (ER) by TAP, which associates with the MHC class I receptor through endogenous antigen presentation pathway specific chaperone: tapasin also known as TAP binding protein (TAPBP) [6, 174]. Co-immunoprecipitation studies in human cells have identified tapasin as a 48 kDa protein associated with TAP, MHC class I and calreticulin [44]. Molecular cloning revealed its identity as a transmembrane glycoprotein containing immunoglobulin superfamily motifs and putative ER retention signal, encoded by a gene that is linked to the MHC [42]. Evidence for its possible function came from a study using the human cell line .220, a lymphoblastoid cell line which does not express tapasin, which demonstrated a lack of any association between MHC class I and TAP, and displayed low levels of MHC class I on the cell surface [175]. The normal phenotype was restored upon transfection with a gene producing recombinant tapasin [42]. Later, studies with tapasin deficient mice showed that not only was the MHC class I surface expression impaired but CD8+ responses during viral infection were also compromised [176, 50].

Additional insights into the functional role of tapasin during antigen presentation came from studies in mammals that focused on its role in optimizing the selection of peptides loaded into the MHC class I receptor, favouring peptides with longer half-life [49, 177, 178], similar to the role of HLA-DM with MHC class II molecules [179]. However the mechanisms by which this role is carried out are still not completely understood. In mammals, the protein levels of tapasin along with other molecules involved in MHC class I assembly such as TAP, β2m and the proteasomal subunits were shown to be induced by the interferon response due to the presence of interferon factor binding elements in the promoter regions of these genes [180, 181].

Tapasin expression in mammals was shown to increase upon infection with the intracellular bacteria: em Listeria monocytogenes and also during treatment with IFNγ in primary embryonic mouse fibroblasts [43]. In salmonid fish, the tapasin gene was shown to be up-regulated during *in vivo* infection with two pathogenic viruses: Infectious salmon anaemia virus (ISAV) and Infectious hematopoietic necrosis virus (IHNV) [86, 122] coordinated increased transcriptional expression of MH class I heavy chain and β2m [99, 98]. Furthermore, tapasin promoter reporter assays demonstrated that tapasin promoters respond to the interferon regulatory factors, Onmy-IRF1 and Onmy-IRF2 [86], which supports its transcriptional activation by the interferon response.

The chum salmon virus (CSV) is a non-pathogenic virus which belongs to the dsRNA aquareovirus family [182, 183]. It has been demonstrated that pre-exposure of rainbow trout to CSV can offer better protection against subsequent infection with IHNV [184]. Later, it was shown that CSV infection can induce the expression of antiviral genes such as MX and vig1 in RTS11 and RTG-2 cells [185], which may contribute to the cells resistance to this virus. In this report, polyclonal antibodies against trout tapasin were developed which allowed the characterization of its protein distribution in trout tissues and cell lines as well as the monitoring of its regulation during poly I:C stimulation and CSV infection in RTS11 cells.

4.2 Materials and methods

4.2.1 Fish

Rainbow trout were obtained from Silver Creek Aquaculture (Erin, ON) and kept in 200 litre fresh-water flow-through tanks at the University of Waterloo under 12:12 h light-dark

cycle. Water temperature was maintained continuously at 13°C. Fish were fed 5 days/week with commercial dry pellet (5 point floating trout chow from Martin Mills, Elmira, ON). All fish were kept and handled under a permit from the University of Waterloo Animal Care committee according to CCAC guidelines. Adult fish (800 $\sim$ grams) were anaesthetised with 1 mL/L of 2-phenoxyethanol (Sigma Aldrich St. Louis, MO). Blood was drawn from the caudal sinus as previously described [149] Tissue samples were collected in RNAlater and stored at -80°.

4.2.2 Cell Lines

RTS11 is a rainbow trout spleen monocyte/macrophage cell line [107] and was maintained at 20°C in Leibovitzs L-15 media (ThermoFisher Scientific, Nepean, ON) with 20% FBS containing 150 U/mL of penicillin and 150 μg/mL streptomycin in 25 cm^2 culture Falcon flasks. RTS11 cells were sub-cultured every 10-14 days by passaging half of the growth-conditioned medium with the cells into new culture vessels containing equal volumes of fresh medium. The following cell lines were maintained in L-15 with 10% FBS in 75 cm^2 culture Falcon flasks and sub-cultured biweekly by trypsin (Lonza, Allendale, NJ). CHSE-214 from chum salmon embryo, RTL-W1 from trout liver [108], RTgill-W1 from trout gill [109], RTgutGC from trout intestinal tract [186]. The salmonid cell lines were grown at 20°C.

Propagation of Chum Salmon Reovirus

Chum Salmon Reovirus (CSV) was obtained from ATCC and routinely propagated on permissible CHSE-214 cultures in L-15 media with 5% FBS at 18°C as previously described [187, 185]. Virus-containing supernatants were collected on day 7 post-infection (p.i.). The supernatants were centrifuged at 4500 $\times$ g for 5 minutes at 4°C, filtered through 0.2 μm membranes (Pall Corporation, Mississauga, ON), aliquotted and frozen at -80°C until used. Viral titers were determined by $TCID_{50}$/mL assays using CHSE-214 reporter cell line as previously described elsewhere [187].

Infection of RTS11 cells with CSV

Approximately 3×10^6 RTS11 cells/well were seeded L-15 with 2% FBS in six well tissue culture plates and subsequently infected with 2.5×10^4 $TCID_{50}$/mL of CSV. Control

cultures had the same number of cells without the virus. Both control and infected cultures were incubated at 14°C for up to 14 days. On selected days post infection cells were collected, washed with ice-cold PBS three times and centrifuged at $500 \times g$ for 4 min at 4°C. Cell pellets were immediately frozen at −80°C. When all samples were collected, cell pellets were thawed on ice and lysed with 100 μL of 1% NP-40 protein containing protease inhibitor (Roche, Mannheim, Germany). Protein concentrations were determined by the BCA protein assay (ThermoFisher Scientific, Nepean, ON).

In vitro stimulation with poly I:C and phyoheamagglutinin

RTS11 cultures were seeded to 2×10^6 in a $25\,cm^2$ flasks and treated for 24 h with 50 μg/mL of Polyinosinic-polycytidylic acid (poly I:C) (Sigma Aldrich St. Louis, MO) or 15 μg/mL of phytohemagglutinin (Sigma Aldrich St. Louis, MO); for controls the same volume of vehicle (PBS) was added. For mRNA analysis, RTS11 cells were treated for 48 h only with poly I:C.

4.2.3 Production of polyclonal antibodies and affinity purification

Rabbit antisera for tapasin were raised against a synthetic peptide conjugated to keyhole limpet haemocyanin (KLH). The peptide was derived from the 21 amino acid C-terminus of trout tapasin (Gene bank ID: AAZ66042.1). Two New Zealand white rabbits (Charles River, ON, CA) were injected intramuscularly with 0.5 mg of tapasin c-terminal peptide with Freund's complete adjuvant (1:1) followed by subsequent peptide boosts of 0.5 mg with Freund's incomplete adjuvant given every three weeks. Rabbits were exsanguinated after 30 weeks and serum was collected and purified using SulfoLink immobilization kit (ThermoFisher Scientific, Nepean, ON) according to manufacturers instruction.

4.2.4 Deglycosylation

Approximately 80 mg of gill tissue was lysed with 1% NP-40 containing 150 mM NaCl, 50 mM Tris [pH 8.0] followed by centrifugation for 15 min at 13,000 rpm at 4°C. Supernatants were collected and concentrated using vivaspin 500 with 3 kDa MWCO (Sartorius, Aubagne, France) according to manufacturers instruction. De-glycosylation with Endo H and N-glycosidase F (New England Biolabs, Ipswich, MA) was performed as previously

described [117]. Enzyme treated and untreated protein lysates were loaded on a 15% gel SDS PAGE gel and blotted with 1:200 anti-tapasin affinity purified antibody.

4.2.5 Western blot

Cell pellets and tissue samples were lysed in 1% NP-40 lysis buffer containing 150 mM NaCl and 50 mM Tris [pH 8.0] supplemented with 1X of protease inhibitor cocktail (Roche, Mannheim, Germany). Thirty μg of protein samples obtained from tissues or cell lysates were separated on a 12% acrylamide gel and transferred to nitrocellulose membranes (Bio-Rad) overnight. Membranes were blocked with 5% skim milk in TBS-T and probed with 1:200 of affinity purified tapasin antibody and with 1:30000 goat anti-Rabbit alkaline phosphatase (Sigma Aldrich St. Louis, MO). Experiments were repeated three times for both cell line and tissue expression.

4.2.6 Reverse transcription and PCR

RNA was extracted from 2×10^6 RTS11 cells using the Qiagen RNeasy extraction kit according to manufacturers instructions (Qiagen, Mississauga, ON) followed by single strand cDNA synthesis using RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific, Nepean, ON). PCR reactions of 25 μL included 1x PCR buffer, 200 μM dNTP mix, 2 μM MgCl2, 600 nM of forward and reverse primer each, 1 μL of RTS11 single strand cDNA, and 1U of Taq Polymerase (MP Biomedical, Solon, OH). PCR was performed at 95°C for 3 min followed by 28 cycles of 95°C 40 sec, 54°C 30 sec, 72°C 90 sec with final extension at 72°C using a BioRad DNA Engine thermocycler (BioRad, Mississauga, ON). Densitometry analysis was performed using IMAGE J platform in which changes in tapasin expression relative to S11 control were measured. The S11 and tapasin primers are listed in Table 4.1.

Gene	**Primer Forward(5'-3')**	**Primer reverse(5'-3')**
Tapasin	AGGATCCATGGCCAATATTTCAACA	GAAGCTTTTACTTCACTTTCTTCTCTT
S11	AGCAGCCAACCATCTTCCAG	ACTCTCCGACGGTAACAATG

Table 4.1: Primers sequences for reverse transcription.

4.2.7 Northern blot

RNA was isolated from both rainbow trout tissues and RTS11 using Trizol according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Briefly, six μg of RNA derived from trout tissues or RTS11 cells were loaded on a 1% agarose-Formaldehyde gel and transferred to a positively charged membrane (Roche, Mannheim, Germany), followed by UV crosslinking. The membrane was hybridized, washed and detected as previously described [149]. The primers used to generate the probes are listed in table 4.2 below:

Tapasin domain	**Primer Forward(5'-3')**	**Primer reverse(5'-3')**
N terminal	CAGAGGGCTCAGTCCATAAACCACAG	TAGCCCCGCTCCCTTTTCCTTGGTCT
Immunoglobulin	CAGAGGGCTCAGTCCATAAACCACAG	AGACCAAGGAAAGGGAGCGGGGCTA
C terminal	GTGAAG GTGCAATGCGAG	TTACTTCACTTTCTTCTCTT

Table 4.2: Primers for tapasin cDNA probes.

4.2.8 Statistical analysis

Statistical analysis was performed using one-way ANOVA and Tukey's *post hoc* test in order to determine if there was a significant change of tapasin protein levels upon *in vitro* stimulation with poly I:C. A probability of $P<0.05$ was considered significant.

4.3 Results

4.3.1 Tapasin protein levels in cell lines and tissues under normal conditions

Examination of the cell line and tissue distribution of tapasin protein confirmed its predominant expression as a 48 kDa band which corresponds with its predicted molecular weight. Among tissues being examined, tapasin protein levels was the highest in peripheral blood leukocytes (Figure 4.3.1A) and in the immune system organs: gill, thymus, head kidney and spleen (Figure 4.3.1B) with the least expression in the heart, muscle and liver (data not shown).

Western blotting analysis from trout cell lines indicated that the protein was predominantly expressed in RTS11 as a 48 kDa band and its expression was also observed in non-immune cell lines such as RTgill-W1 and RTL-W1 with the least expression in RTgutGC

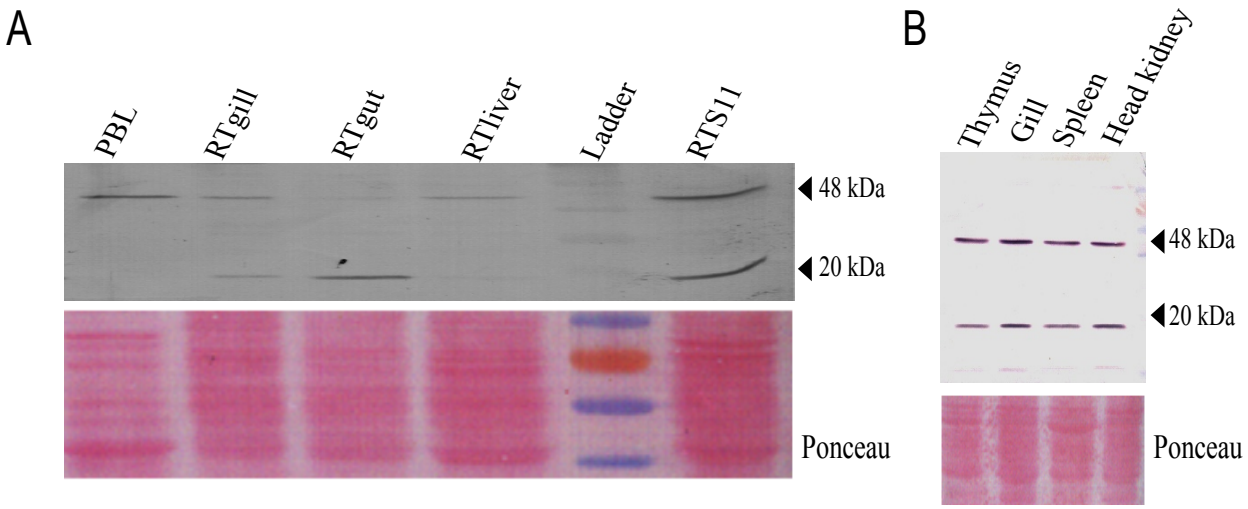

Figure 4.3.1: **Western blot detection of tapasin protein in rainbow trout cell lines and tissues under non stimulating conditions.**
Protein distribution of tapasin in unstimulated trout cell lines (A) and in unstimulated tissues (B), showing the detection of two distinct bands: the predicted 48 kDa protein and an additional 20 kDa. The position of the molecular mass markers is shown on the right margin.

(Figure 4.3.1A). These results correlated with the transcriptional profile previously found in trout and salmon, however, they did not correspond to high mRNA levels found in both heart and gut [79, 122]. Interestingly, in western blots of cell lines and tissues an additional 20 kDa band was observed with relatively high expression in the RTgutGC cell line and in tissues with immune functions (Figure 4.3.1B) whereas in PBL the 20 kDa band was undetected.

4.3.2 Tapasin transcriptional expression

In order to investigate whether or not the 20 kDa band could be a result of alternative splicing of the tapasin gene, three separate northern blot probes were designed to span the N-terminal, immunoglobulin and C-terminal domains of trout tapasin, respectively. When the immunoglobulin probe was used, only a single transcript band was detected (Figure 4.3.2). An additional smaller sized band was detected at lower expression levels with the N- and C-terminal probes.

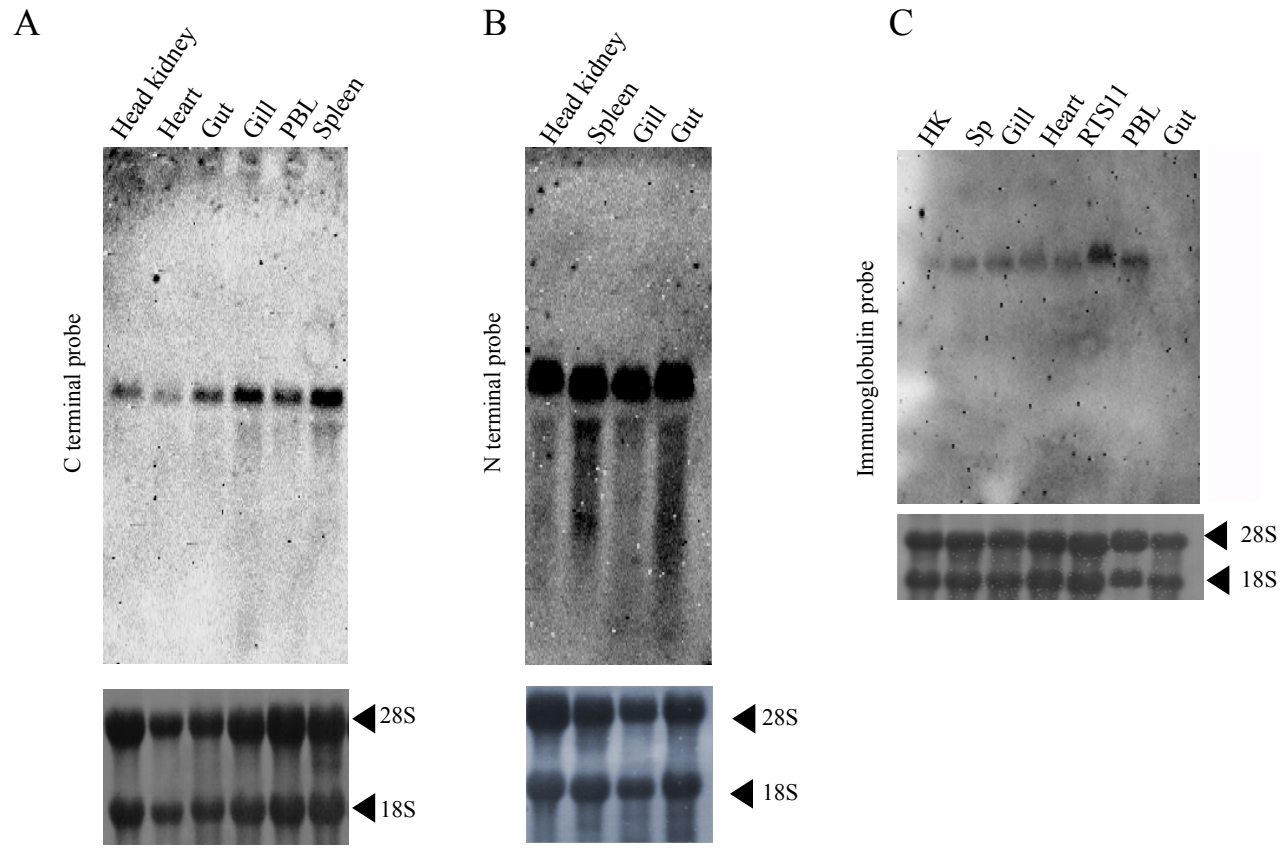

Figure 4.3.2: **Northern blot showing the tissue distribution of trout tapasin transcript under naive conditions in rainbow trout.**
Distribution of trout tapasin transcripts using three distinct probes for: the C terminal domain (A), the N terminal domain (B) and the immunoglobulin domain (C). The arrows on the right margin show the size of the 28S and 18S ribosomal bands.

4.3.3 N-glycosylation of Tapasin

The trout and salmon tapasin proteins both have a putative, conserved N-glycosylation site. In humans, tapasin has been shown to have a single glycosylation site which aligns with with trout tapasin at position 252, and mutation of this site in human tapasin has been shown to reduce the recruitment of calreticulin to the peptide loading complex [188]. To test if the predicted N-glycosylation site of tapasin corresponds to functional glycosylation *in vitro*, lysates from trout gill were treated with EndoH and N-glycosidase F then examined for a shift in tapasin protein size on SDS-PAGE. As seen in Figure 4.3.3, tapasin protein is subjected to N-type glycosylation in the gill tissue, as indicated by its molecular size shift compared to the mock treated sample.

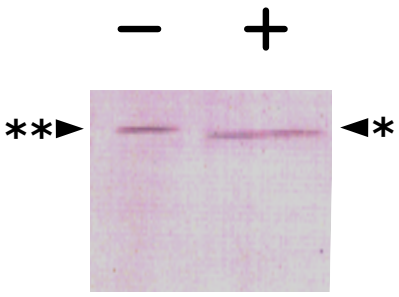

Figure 4.3.3: **Western blot analysis of tapasin protein in RTS11 following de-glycosylation.** Tapasin protein in the gill following deglycosylation (+) and reaction containing no enzyme (-). The mobility shift after treatment indicates that tapasin is N-glycosylated. The protein mobility with no enzymatic treatment is labeled with two asterisks whereas the protein mobility after enzymatic treatment is labeled with a single asterisk.

4.3.4 Tapsasin protein levels upon immunostimulation and viral infection

To explore the regulation of tapasin protein under interferon mediated conditions, RTS11 cells were stimulated with poly I:C, to mimic a viral infection [189, 190, 121] and monitored for protein expression levels after 24 h. As demonstrated in Figure 4.3.4A, poly I:C stimulation in RTS11 cells induced the expression of tapasin mRNA after 24 h which correlated with a 3.5 fold increase at the protein level compared with the control (Figure 4.3.4B). To test if this upregulation corresponds to a similar induction of the MH class I, RTS11 were stimulated with poly I:C and monitored for the expression of the MH class I heavy chain, known to be glycosylated in the trout [117] and in RTS11 (data not shown).

As seen in Figure 4.3.5, the glycosylated form of the heavy chain was upregulated up to 4 fold in poly I:C treated cells indicating active and enhanced maturation of the MH class I in addition to upregulation of the amount of protein, whereas no change was observed for other MH class I bands, which may be unglycosylated intermediates and/or other allelic forms of the MH class I heavy chain recognized by the antibody. To further study the regulation of tapasin by dsRNA, RTS11 cells were infected for 14 days with the dsRNA chum salmon virus (CSV) [191] and monitored for the expression of tapasin at different time points both at the protein and transcriptional level. As demonstrated in Figure 4.3.6A, tapasin protein is upregulated up to 3 fold compared to the control by day 14 post infection, and this observation was supported by increased transcript levels in infected cells (Figure 4.3.6B). The co-regulation of tapasin and the MH class I protein was evident by a coordinated increase in glycosylated MH class I heavy chain during infection

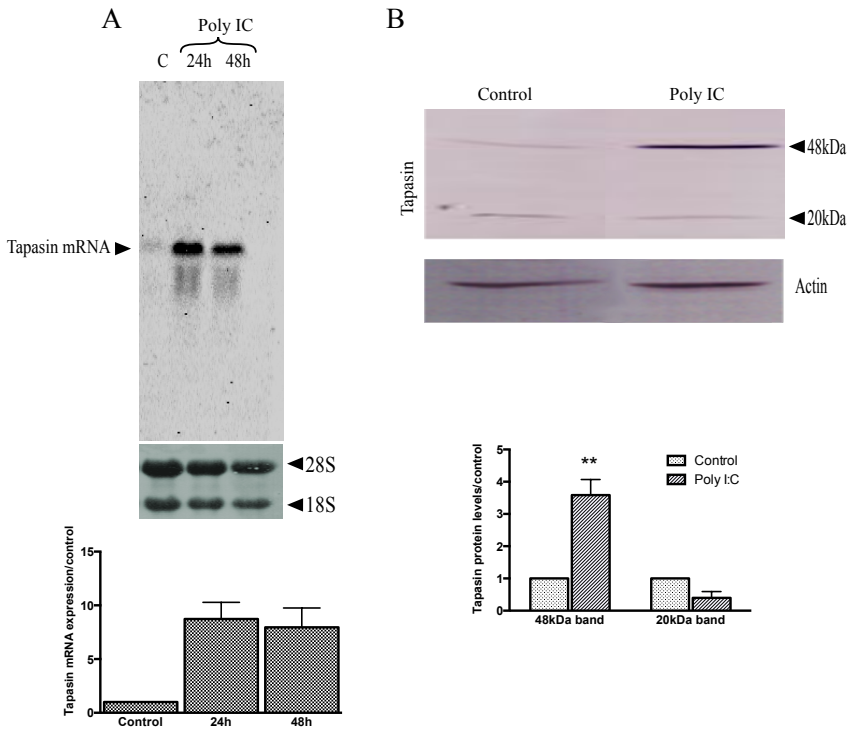

Figure 4.3.4: **Tapasin transcript and protein levels in RTS11 cell line after *in vitro* induction with poly I:C.**
A) Northern blot analysis showing tapasin mRNA expression level in RTS11 after 48 h of *in vitro* induction with 50 μg/mL of poly I:C. The arrows on the right margin show the estimated size of the 28S and 18S ribosomal bands. B) Tapasin protein levels in the RTS11 cell line after 24 h of *in vitro* induction with 50 μg/mL of poly I:C. The graph in A demonstrates the ratio between tapasin transcript and the 28S ribosomal bands and is representative of two separate experiments. The graph in B shows the ratios between the tapasin and actin bands and are representative of three separate experiments. Error bars represent the standard error. Significant differences between the control and treatment groups are represented by ** ($p<0.003$).

which peaked up to four fold 14 days post infection (Figure 4.3.7). In contrast to the induction of the 48 kDa tapasin protein, a decrease in expression levels were observed for the 20 kDa band during poly I:C treatment (Figure 4.3.4B), while no overall change in expression was detected for the 20 kDa band during CSV infection (data not shown).

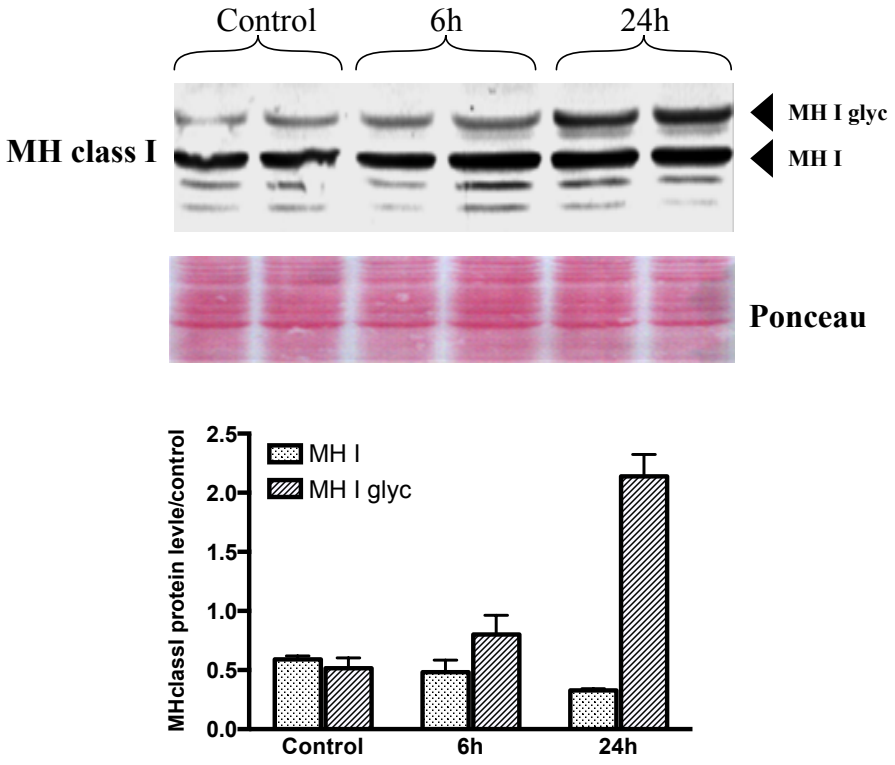

Figure 4.3.5: **MH class I protein levels in RTS11 after 48h of *in vitro* induction with poly I:C in RTS11 cells.**

Western blot analysis demonstrating the protein levels of the MH class I after 48 h of *in vitro* induction with 50 μg/mL of poly I:C. Double lanes for each time point represent loading of two separate experimental repeats. The position of the MH class I glycosylated (MH I–glyc) and the main lower MH I bands are indicated by the arrowheads on the right margin. Densitometry analysis was conducted using image J software (version 1.44). Graphs show the ratio between MH class I and the prominent Ponceau stain band. Bars represent the standard error. These results are representative of two separate experiments.

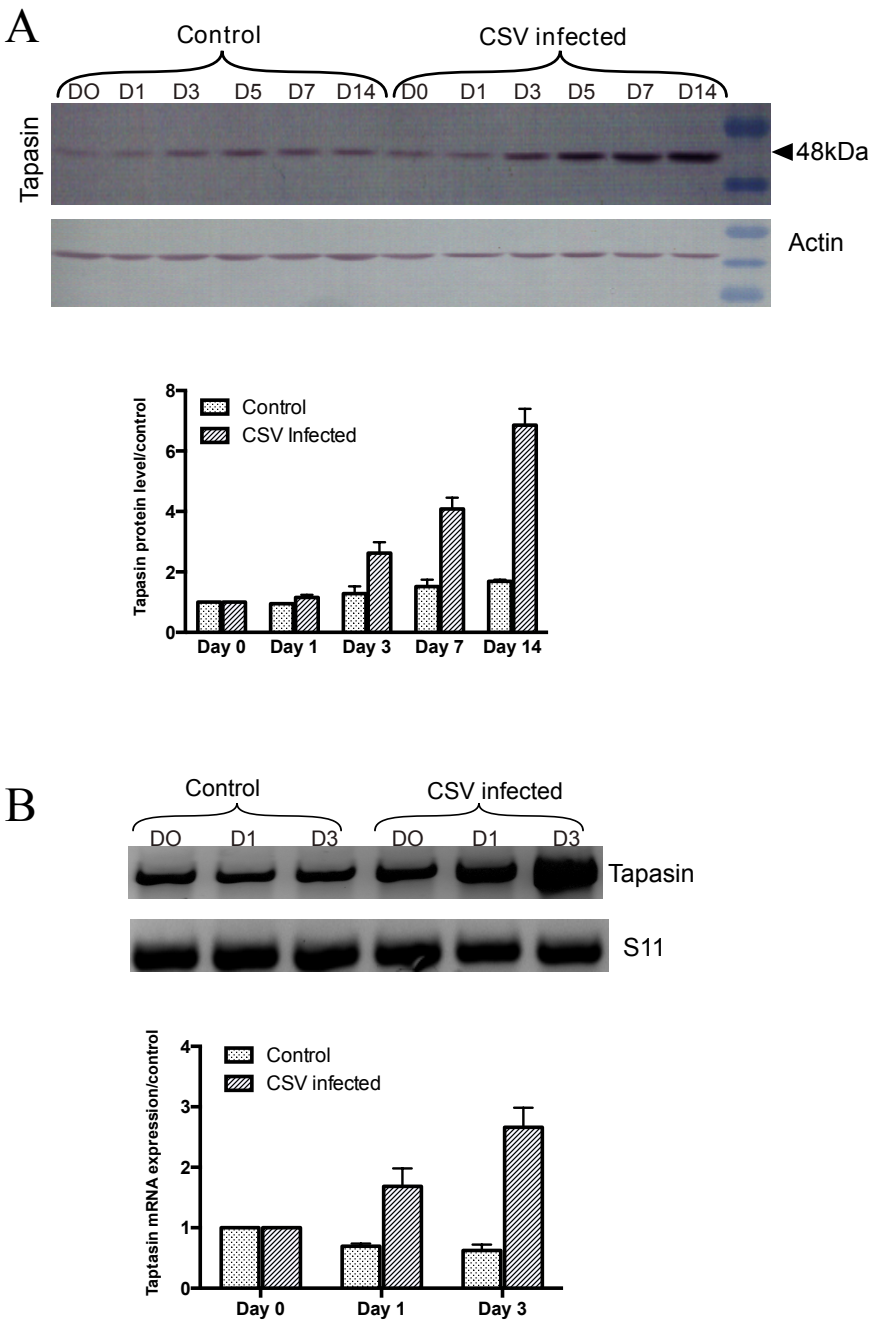

Figure 4.3.6: **Tapasin protein levels and transcript expression in RTS11 infected with chum salmon virus.**

A) Tapasin protein levels in CSV infected RTS11 for 14 days. Densitometry analysis was conducted using image J software (version 1.44). Graphs represent the ratios between the tapasin and the actin protein bands and bars represent the standard error. These results are representative of two separate experiments. B) RT PCR analysis showing tapasin transcript expression in RTS11 cells following three days of CSV infection. Results are expressed as the ratios between the tapasin and S11 protein amplicons. The vertical bars represent the standard error. These results represent two separate experiments.

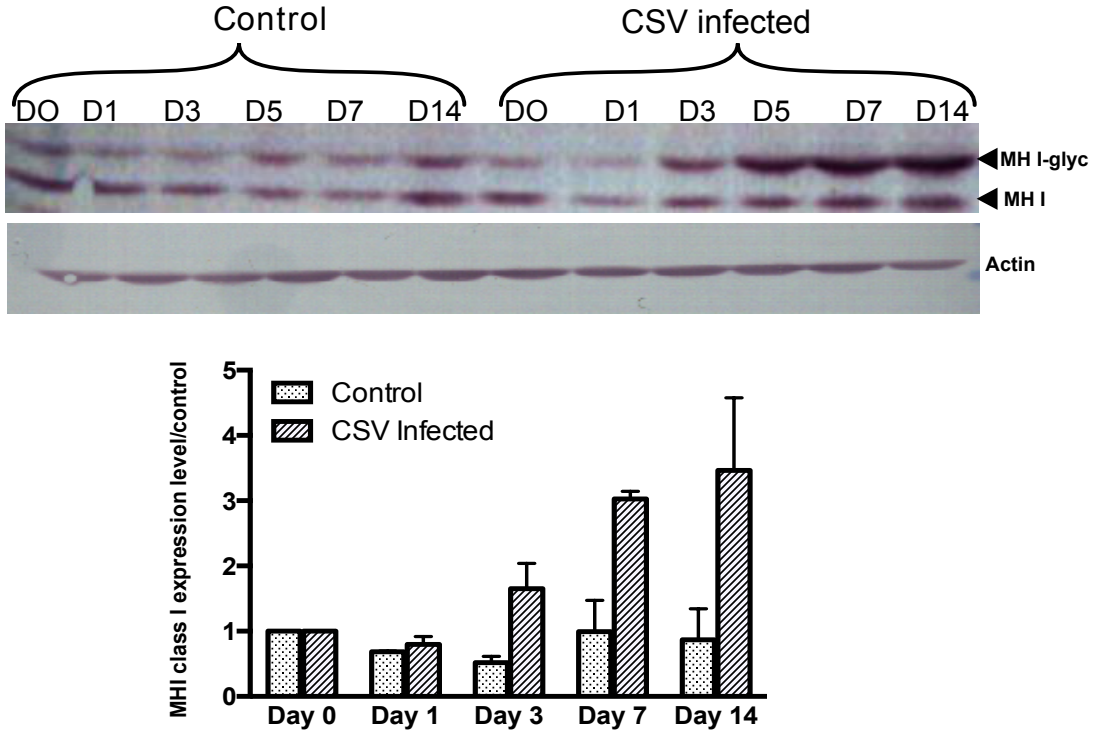

Figure 4.3.7: **MH class I protein levels in RTS11 infected with chum salmon virus.** Western blot analysis showing the MH class I protein levels in RTS11 infected with CSV for 14 days. The position of the MH class I glycosylated (MH I-glyc) and the additional lower MH I band are indicated by the arrowheads on the left margin. The graph in the lower left panel shows the ratio between the glycosylated MH class I and actin protein bands. Vertical error bars represent the standard error. These results are representative of two separate experiments.

4.4 Discussion

The tapasin gene has been previously characterized in teleost fish [86, 122, 192, 193] and, in rainbow trout, has been shown to reside within the MH class I region where it has promoter elements responding to interferon regulatory factors [86]. Interestingly, in trout there are two gene copies for tapasin: a truncated version missing most of the immunoglobulin domains, transmembrane domain and the C terminus (TAPBP.b) that is located on the class Ib region between MH class I UCA and UDA and a full length version (TAPBP.a) which is linked to the class Ia locus together with other antigen processing genes [86]. The full length teleost tapasin shares common features with human tapasin such as a predicted leader peptide, immunoglobulin superfamily domains, transmembrane domain and an ER retention signal as well as a single potential glycosylation site. Unlike other trout ER chaperones such as calreticulin, ERp57 and calnexin that share high sequence identity with their mammalian orthologues, the trout tapasin protein sequence is much less conserved with only 30% identity.

4.4.1 Tapasin protein levels under naive conditions

To study tapasin protein distribution and its regulation during viral infection, polyclonal antibodies were developed against the C-terminal domain of tapasin, the domain known to associate with TAP. Cell line and tissue expression patterns demonstrated that the trout version of this protein is detected as a 48 kDa N-glycosylated band that is primarily expressed in cells and tissues of the immune system. Furthermore, an additional 20 kDa band was also detected in the cell lines and tissues, with the highest expression in RTgutGC while no expression of this form was observed in peripheral blood leukocytes. Interestingly, evidence for tapasin alternative splice variants was recently described in human melanoma cell line, which contained transcripts with a deletion of exon three encoding a variant protein that interacts with TAP, however could not support the MHC class I assembly and surface expression on the cell surface when transfected into tapasin deficient cell lines [194].

4.4.2 Tapasin transcript distribution in trout

To explore the possibility of a trout alternative splicing variant which might produce the 20 kDa protein, three separate northern blot probes were generated against the proxi-

mal, middle and distal domain of trout tapasin. Our results demonstrate the presence of one main transcript for tapasin in all trout tissues, with a minor band detected by the C-terminal and N-terminal probes, suggesting a splice product missing the immunoglobulin domains in a manner similar to the human melanoma specific form mentioned above, although the proportion of the minor transcript does not match the proportion of total protein represented by the 20 kDa band. Additionally, RACE experiments aimed at isolating a variant transcript sequence failed to produce a shorter tapasin transcript and are being further explored. The other possibility could be a different gene for tapasin, similar to TAPBP.b, with a unique promoter. It is possible that the trout TAPBP.b gene which codes for a transcript with deleted 3' end [86] may encode the 20 kDa protein band. In this case however, it would have to contain the last 21 amino acids of tapasin since that is the epitope the antibody recognises. However, Landis et al. [86] were unable to identify a transcript from this gene that contained that sequence. Thus, isolation of the full length 3' coding sequence of TAPBP.b is necessary to validate if this gene encodes for a protein with a cytoplasmic tail that could be this 20 kDa form of tapasin.

4.4.3 Tapasin protein expression following dsRNA stimulation and CSV infection

Human genes that participate in the antigen presentation pathway, which include the MHC I, β2 microglobulin, TAP1, TAP2, and tapasin are transcriptionally induced by interferon gamma (IFNγ) [43, 195, 196, 197]. Sequence analysis of the mammalian and trout tapasin promoter revealed consensus binding sites for interferon regulatory factor, IRF-1 and IRF-2 [86, 195, 181, 198] that contributes to the transcriptional induction of tapasin during the activation of the immune response. In this study, the expression of tapasin protein was investigated upon treatment with the synthetic dsRNA poly I:C and during infection with CSV in RTS11 cells. Expression levels of tapasin protein were enhanced up to 3.5 fold after 24 h of poly I:C treatment, which correlated with an approximate 9 fold increase in transcript levels. In Atlantic salmon kidney cells, the transcript expression of tapasin measured by real time PCR was shown to increase only up to 3.5 fold after 24 h of treatment with 10 μg/m of poly I:C, however expression continued to increase until three days post stimulation where it peaked at 15 fold which further supports the conserved transcriptional activation of tapasin by synthetic double stranded RNA [122]. The mechanism involved in tapasin induction in RTS11 was shown to possibly involve the IFNγ pathway that is known to be exclusive to immune system cells [92]. A microarray analysis that characterized the

transcriptional response of RTS11 after 6 hours of recombinant IFNγ treatment demonstrated a strong induction of IRF-1 and class I pathway genes, among which tapasin was most strongly expressed, whereas the fold increase of the MH class I transcript was found to be relatively low at this time point [199]. Here, upon stimulation with both poly I:C and CSV, the protein levels of tapasin protein reached to higher levels compared to MH class I. This strong upregulation can act to rapidly increase tapasin protein level, which is relatively low levels under constitutive conditions.

Interestingly, previous studies in mammalian cells showed that the induction of TAP1 by cytokines such as IFNβ and IFNγ was significantly more rapid than the MHC class I both at the mRNA and the protein level [200], however the induction of the mammalian tapasin appears to be slower than TAP1 and LMP [43], which may not be the same case for fish, based on the results at the transcript level [122, 199]. Interestingly, no change was observed in the expression of the 20 kDa tapasin band during treatment with dsRNA or chum salmon viral infection, which implies that full length tapasin protein and the 20 kDa protein band are differentially regulated and possibly play different roles in the immune response. In summary, this report demonstrates the cell line and tissue distribution of trout tapasin protein and shows for the first time the induction of tapasin protein during CSV infection and following poly I:C treatment which supports its conserved function in the assembly of the MH class I. Further research will aim to reveal tapasins interaction with the MH class I as part of the MH class I peptide loading complex in fish and elucidate the identity and function of the 20 kDa tapasin variant.

Chapter 5

Induction of MH class I and accessory proteins by viral haemorrhagic septicaemia virus

5.1 Introduction

The MHC class I receptor is expressed on the cell surface of all nucleated cells in vertebrates and is responsible for the display of foreign peptide fragments derived from intracellular pathogens. In order to assure the stability of the MHC class I receptor during export and on the cell surface, a stringent and specialized MHC class I pathway has evolved in the ER to facilitate the optimal folding of the MHC class I with a diverse pool of peptides. This process is executed in mammals by the assembly of the peptide loading complex which involves multiple protein interactions that includes the heterodimer MH class I-β2m, ERp57, tapasin, calreticulin and an endogenous peptide [6, 174, 8].

The first teleost major histocompatibility (MH) gene fragments were isolated from carp [60] and later genomic linkage studies mapped a unique architecture in which the MH class I and class II loci do not reside on the same chromosome as found in mammals. However, the genes for the proteasomal subunits: low molecular mass polypeptide low molecular mass polypeptide (LMP2, LMP2/δ), TAP heterodimer and tapasin (TAPBP.A) are linked to the teleost class Ia locus [83, 84], which highlights their role as a functional co-operative unit in antigen presentation. in mammals, genes encoding the class I pathway are regulated by the action of the interferons (IFN), which are rapidly induced during viral infections. Receptor

binding of interferon on the cell surface of both immune and non-immune cells activates a JAK-STAT pathway which subsequently turns on the expression of many interferon stimulated genes (ISG) including the MHC class I pathway genes [93, 94]. Studies in salmonid fish have demonstrated a similar interferon mediated response that results in the transcriptional induction of the MH class I pathway during in vivo infections with infectious hematopoietic virus (IHNV) and infectious salmon anemia virus (ISAV) [98, 99]. In addition, promoter analysis of these genes in trout revealed putative regulatory factor (IRF) and IFNγ binding site (GAS) supporting their activation by interferon [98, 99].

Viral haemorrhagic septicaemia virus (VHSV) is the cause of a fish viral disease that affects both freshwater and marine species in the northern hemisphere, causing major economic impact in cultured species such as rainbow trout [201]. Classified in the Novirhabdovirus genus within the Rhabdoviridae family, VHSV contains a non-segmented, single stranded RNA genome that encodes five basic structural proteins comprising nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), RNA polymerase (L), and a single non-structural protein, called non-virion (NV) protein [202]. Four major genotypes of VHSV have been identified based on phylogenetic tree analysis of the N and the G sequences: I, II, III and IV [203]. The genotype IV is further subdivided into VHSV IVa and VHSV IVb in which IVa genotype is endemic to a wide range of pacific fish species and cause low mortality in rainbow trout [204].

Although, much progress has been made in recent years in understanding the anti-viral mechanisms against this virus both *in vitro* and *in vivo*, little is known about the possible temperature regulation of the MH class I antigen presentation machinery at the protein level during the course of rhabdoviral infection. Interestingly, a study conducted with the murine lymphoma RMA-S cell line demonstrated that at a low temperature (26°C) relative to murine cells, MHC class I molecules can dimerize with β2m, and exit the Golgi to the plasma membrane, but do not contain a bound peptide and therefore fail to present antigen to cytotoxic T cells [100]. This finding raises an interesting question about how antigen presentation is maintained during viral infection in poikilothermic animals, such as bony fish, as their physiology and immune response change in response to their environmental temperature.

It has been previously demonstrated in carp that expression of class I heavy chain–β2m, heterodimer is abolished at 6°C in PBL, while surface expression of immunoglobulin on the surface of B cells remained unchanged [205]. This study showed that surface expression of the MH class I molecule can be restored after 6 days when the fish are returned to

12°C and that the lack of expression at 6°C was mediated by down regulation of β2m transcription [205]. However, a study with rainbow trout and atlantic salmon showed that the cellular levels of β2m can be maintained at 2°C in both species and indicated on a persistent surface expression of β2m in PBL at this temperature [101].

Monocyte/macrophage cell lines have been useful tools to study many aspects of immunology including antigen processing and presentation. The monocyte/macrophage RTS11 cell line was developed from the spleen of rainbow trout and demonstrated characteristics of antigen-presenting cells [206, 185, 199, 79, 107, 207]. Thus in this study, RTS11 cell line was used to study the effect of temperature on the regulation of MH class I antigen presentation in response to VHSV IVa both at 14°C and 2°C to better understand antigen presentation mechanisms responsible for resistance to infection at cold temperature.

5.2 Materials and methods

5.2.1 Fish and VHSV IVb infection trial

Rainbow trout (10–15 g) were obtained from Lyndon Fish Hatcheries (New Dundee, ON) and kept at 13°C in 60 L tanks in a single–pass well water at the University of Guelph under 12:12 h light-dark cycle. Fish were fed with commercial dry pellet (Martin Mills, Elmira, ON) in a ration of 1% of their body weight. Experimental infections with VHSV IVb were conducted in rainbow trout (n=21) for 14 days. Fish were injected intraperitoneally with 100 μL of 1×10^7 $TCID_{50}$/mL of VHSV IVb. Three fish were used as a negative control and injected i.p. with 100 μL of minimum essential Medium (MEM). Fish were sacrificed at various time points and tissues from the gill, spleen and head kidney were collected in RNAlater and kept at -80°C until later use. Animals were kept according to the guidelines under the approval of the University of Guelph. Virus isolation and propagation was performed as previously described [208].

5.2.2 Fish Cell Lines

The rainbow trout spleen monocyte/macrophage cell line RTS11 [107] was grown in Leibovitzs L-15 medium (ThermoFisher Scientific, Nepean, ON) with 20% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin (ThermoFisher Scientific, Nepean, ON) in 25 cm^2 culture flasks at 20°C and sub-cultured every 10-14 days. The EPC cell line derived from

fathead minnow [209] was maintained in L-15 medium with 10% FBS and antibiotics in 75 cm^2 culture flasks at 26°C and sub-cultured every 7 days by using trypsin (Lonza, Allendale, NJ).

5.2.3 Propagation of VHSV

VHSV genotype IVa isolated from Pacific herring [210] was routinely propagated on permissible EPC cultures in L-15 with 2% FBS at 14°C. Virus-containing supernatants were collected on day 10 when severe cytopathic effects were observed. The supernatants were centrifuged at 4500 × g for 5 minutes at 4°C, filtered through 0.2 μm membranes (Pall Corporation, Mississauga, ON), aliquoted and frozen at −80°C until used. Viral titers were determined by $TCID_{50}$/mL assays using EPC cell line for VHSV IVa, as previously described [207].

5.2.4 Infection of RTS11 cells with viruses

L-15 with 2% FBS was used for all infection experiments. Approximately 3×10^6 RTS11 cells/well were seeded in six-well tissue culture Falcon plates and subsequently infected with 1.5×10^7 $TCID_{50}$/mL of VHSV IVa at either 2°C or 14°C for 14 days. Control cultures had the same number of cells in L-15 medium with 2% FBS without virus. Cells were harvested on selected days, washed with ice-cold PBS twice and then pelleted at 500 × g for 4 min at 4°C and immediately frozen at −80°C until all samples were collected.

5.2.5 *In vitro* stimulation with poly I:C

About 2×10^6 RTS11 cells were seeded in L-15 with 10% FBS into 25 cm^2 Falcon flasks and treated for 72 h with 50 μg/mL of polyinosinic-polycytidylic acid (poly I:C) in PBS (Sigma Aldrich, St. Louis, MO). For control samples the same amount of vehicle was added.

5.2.6 Western blot

Cell pellets and tissues were lysed in 1% NP-40 lysis buffer containing 150 mM NaCl and 50 mM Tris [pH 8.0] supplemented with 1x of protease inhibitor cocktail (Roche, Mannheim, Germany). Protein concentrations were determined by the BCA protein assay

(ThermoFisher Scientific, Nepean, ON). Cell lysates were separated on a 12% acrylamide gel and transferred to nitrocellulose membranes (Bio-Rad, Mississauga, ON) overnight. Membranes were blocked with 5% skim milk in TBS-T and probed with rabbit polyclonal antibodies raised against trout recombinant proteins as follows: MH class I heavy chain (1:500), MH class IIβ (1:500), β2m (1:500) ERp57 (1:1000), calreticulin (1:500) and tapasin antibody (1:200) generated against the C-terminal domain of trout tapasin. Membranes were subsequently probe with 1:30000 goat anti-rabbit alkaline phosphatase (Sigma Aldrich, St. Louis, MO) and detected using NBT/BCIP (Roche, Mannheim, Germany) according to the manufacturers instruction. Densitometry analyses of the MH class I heavy chain was conducted by image J software (version 1.44) only for the MH class I glycosylated band, which represents the native and active form of this protein.

5.2.7 Reverse transcription and PCR

RNA was extracted from 2×10^6 RTS11 cells using RNeasy extraction kit according to manufacturers instructions (Qiagen, Mississauga, ON) followed by single strand cDNA synthesis using a Fermentas RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific, Nepean, ON). The PCR reaction of 25 μL included 1x PCR buffer, 200 μM dNTP mix, 2 mM MgCl2, 1 μL of RTS11 single strand cDNA template and 1 U of Taq Polymerase (MP Biomedical, Solon, OH). PCR was performed at 95°C for 3 min followed by 28 cycles of 95°C 40 sec, 54°C 30 sec, 72°C 90 sec, with final extension at 72°C using a BioRad DNA Engine thermocycler (BioRad,Mississauga, ON). Primers used are indicated in Table 5.1 below:

Gene	**Primer Forward(5'-3')**	**Primer reverse(5'-3')**
MH class I	AAGCTTAACATGAAGGGTATTATCTTG	CGGCCGGAGGTCTCTCAAATCTTCTGG
Tapasin	AAGCTTATGTCGATGCCAATT	CGGCCCGATTCTAGAGCACCCCAGAAGAC
β2m	AAGCTTATGAAGTCTATTCTGTTAATC	GAATTCCCAGATCCTTACATATCTGC
S11	AGCAGCCAACCATCTTCCAG	ACTCTCCGACGGTAACAATG

Table 5.1: Primers sequences for reverse transcription.

5.2.8 Northern blot

RNA was isolated from head kidney and gill using Trizol according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Six μg of RNA derived from trout tissues

was loaded on a 1% agarose-formaldehyde gel and transferred to a positively charged membrane (Roche, Mannheim, Germany), followed by UV crosslinking. The membrane was hybridized, washed and detected as previously described [149]. The primers used to generate a cDNA probe for the Ig domain of tapasin were as follows: forward 5' CAGAGGGCTCAGTCCATAAACCACAG 3' and reverse 5' AGACCAAGGAAAGGGAGCGGGGCTA 3'.

5.2.9 Detection of β2 microglobulin accumulation in conditioned media

Approximately 6×10^6 RTS11 cells in L–15 with 2% FBS were seeded per well in six well tissue culture plates and subsequently inoculated with 300 μL of 1.5×10^9 $TCID_{50}$/mL of VHSV IVa. To ensure the same final volume, 300 μL of fresh L–15 with 2% FBS was added into control cultures. Both control and virally infected cultures were incubated at 14°C for up to 14 days. Conditioned media was collected in 15 mL conical tubes and centrifuged at $500 \times g$ for 4 min at 4°C to remove cells. The supernatants were syringe-filtered through 0.2 μm low-protein-binding membranes (Pall Corporation, Mississauga, ON). The flow-through media were subsequently frozen at −20°C until all samples were collected. Cell pellets were washed with ice-cold PBS twice and re-suspended in 1 mL of ice cold PBS on ice. Seventy five microliters of the cell suspensions were taken to determine the cell concentrations by the Moxi ZTM Mini Automated Cell Counter (Orflo Technologies, Hailey, ID); each sample was counted twice. Cell suspensions were then pelleted at $1000 \times g$ for 3 min at 20°C. Cell numbers were determined for each time point by the Scepter 2.0 handheld automated cell counter (Millipore, Billerica, MA) and cell pellets were stored at −80°C for western blotting to show β–actin protein levels.

5.2.10 Immunocytochemistry

Five hundred μL of 2×10^5 RTS11 cells were plated in L-15 with 2% FBS in four chamber slides (Millipore, Billerica, MA). Infected cultures were treated with 50 μL of 1.5×10^9 $TCID_{50}$/mL of VHSV IVa at 14°C. After 7 days, cultures were gently washed with PBS once and then fixed in 0.25% paraformaldehyde for 20 min at 4°C. Both infected and non-infected cultures were blocked in 1% bovine serum albumin in PBS for one hour and then probed with rabbit affinity-purified anti-trout MH Iα at 1:50 in blocking buffer

overnight. After washing for three times with PBS, cultures were probed with AlexaFlour 488-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA) at 1:3000 in PBS for one hour. To determine unspecific binding, both infected and non-infected cultures were probed with secondary antibody only. All cultures were gently washed with PBS three times. Slides were allowed to dry at room temperature and then mounted with Fluoroshield with DAPI (Sigma Aldrich, St. Louis, MO) for 10 minutes.

5.2.11 Statistical analysis

Statistical analysis was performed using one-way ANOVA and Tukeys post hoc test. A probability of $P<0.05$ was considered significant.

5.3 Results

5.3.1 Expression of MH class I and associated chaperones in poly IC stimulated RTS11

Poly I:C, is a double stranded RNA mimic known to induce anti-viral state in fish through the induction of interferons (IFNs) [190, 211, 212, 121]. To explore the regulation of the MH class I during interferon stimulating conditions, RTS11 cells were induced with 50 μg/mL of poly I:C, a concentration previously shown to upregulate β2m at the protein level in RTS11 cells [79].

As shown in Figure 5.3.1, the MH class I heavy chain is detected as two similar sized 45 kDa bands which represent the two forms of the MH class I heavy chain: the glycosylated (upper band) and non glycosylated (lower band) [117], with some minor bands which represent the other allelic forms of the protein. The glycosylated heavy chain band was enhanced by poly I:C stimulation whereas no change was observed for the other MH class I protein bands. The MH class I heavy chain, β2m and tapasin were upregulated after three days of treatment up to 2.5, 3.2 and 5.3 fold respectively, which demonstrates that their relative induction by poly I:C varies in magnitude. In contrast to the increased levels of MH class I, no change was observed in the protein levels for ERp57 and calreticulin.

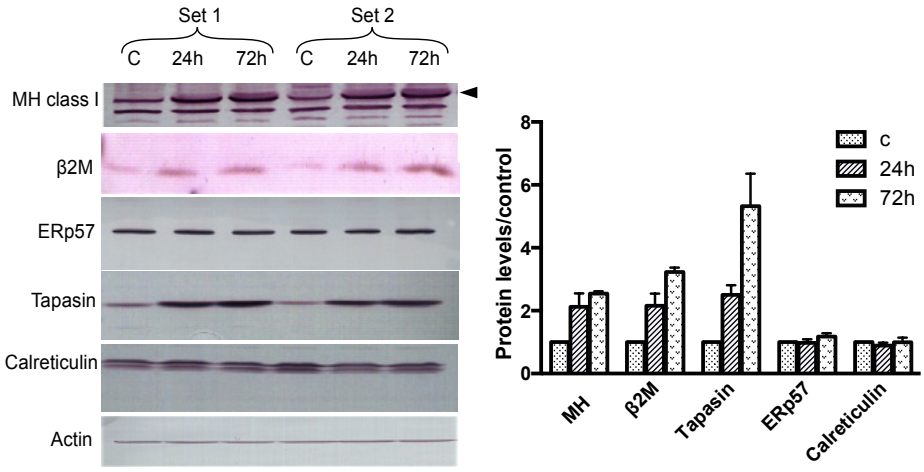

Figure 5.3.1: **Protein levels of the MH class I machinery after *in vitro* induction with poly I:C in RTS11 cells.**
Western blot analysis showing protein levels of MH class I heavy chain, β2m and associated chaperones in RTS11 upon treatment with 50 μg/mL of poly I:C for 72 h. The arrowhead represents the position of the glycosylated MH class I band. Densitometry analysis was conducted using Image J software and results are expressed as the ratios between the MH class I complex proteins and the actin bands. These results are representative of two separate experiments. Bars represent the standard error.

5.3.2 Expression of MH class I and associated chaperones in VHSV IVa simulated RTS11 at 14 degrees

In order to study the expression of the MH class I pathway proteins in response to rhabdovirus infection, RTS11 cells were infected with VHSV IVa. As shown in Figure 5.3.2, while inducible expression of the MH class I heavy chain and β2m, was observed, no change in the overall expression was observed for MH class II protein, perhaps demonstrating the importance of the MH class I pathway versus the MH class II response during the early stages of VHSV infection. MH class I protein expression in viral infected cells increased up to 1.8 fold on day 3 followed by a 4.6 fold increase on day 14. The increased protein level of MH class I were also observed during VHSV IVb infection *in vivo* which indicated on upregulation of the MH class I starting from day 3 p.i. up to day 14 in the gill tissue (Figure 5.3.3).

Similarly to poly I:C stimulation, no change in expression levels was observed for ERp57 and calreticulin whereas tapasin expression levels were induced to highest level on day 14 (Figure 5.3.4) post infection (p.i.). Furthermore, at the transcript level, tapasin was induced up to six fold three days p.i., which was shown to be in line with the upregulation

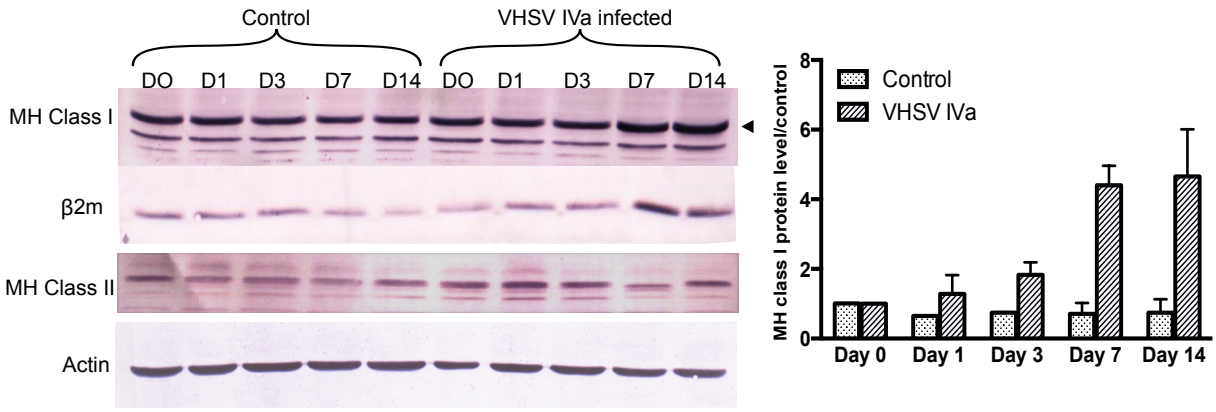

Figure 5.3.2: **Western blot analysis showing protein levels of MH class I, MH class IIβ and β2m in RTS11 cells during VHSV IVa infection at 14 degrees.**
Protein levels of MH class I heavy chain, β2m in RTS11 using control cells and cells infected with VHSV IVa at 14 degrees for 14 days. Densitometry analysis for MH class I heavy chain was conducted using Image J software. Bars represent the standard error. These results are representative of two separate experiments. The position of the glycosylated MH class I band is represented by the arrowhead.

seen at the protein level. Increased levels of tapasin were also observed during infection of rainbow trout with VHSV IVb in both head kidney and gill tissues which indicate that induction of tapasin during VHSV infection also occurs *in vivo*.

Interestingly, no change in the transcript expression was observed for β2m and only a 1.3 fold increase was observed for the MH class I heavy chain (Figure 5.3.5), suggesting on their later induction at the transcriptional level during VHSV IVa infection.

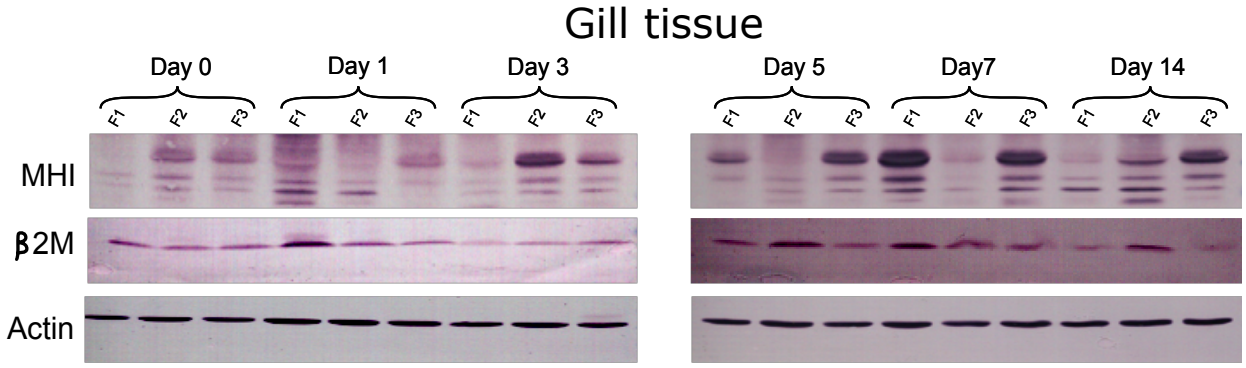

Figure 5.3.3: **Western blot analysis showing protein levels of MH class I heavy chain and β2m in rainbow trout gill tissue upon *in vivo* infection with VHSV IVb.**
Rainbow trout were infected with VHSV IVb for 14 days and monitored for the protein levels of MH I and β2m in gill tissue on day 0 (D0), day 3 (day 3) and day 7 (D7). Day 0 samples were obtained from a negative control fish. Tissue lysates for each time point were obtained from three individual fish (F) and used as triplicates.

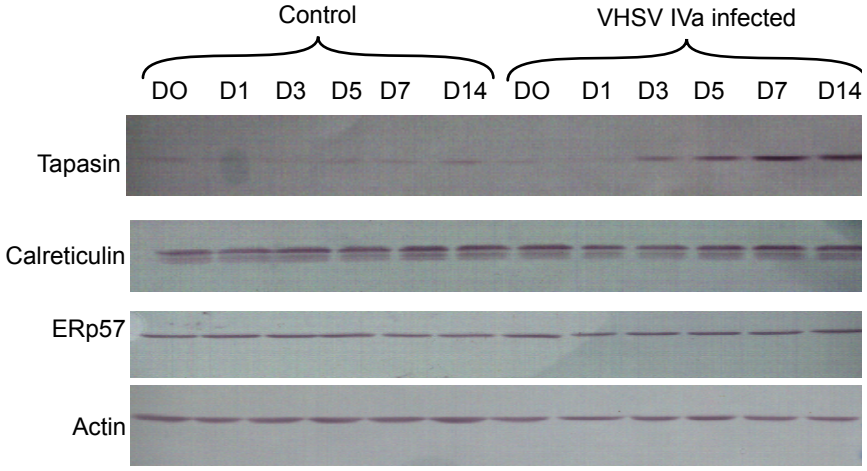

Figure 5.3.4: **Western blot analysis showing protein levels of ERp57, calreticulin and tapasin in RTS11 during VHSV IVa infection.**
Protein levels of ERp57, calreticulin, tapasin in RTS11 using control cells and cells infected with VHSV IVa at 14 degrees for 14 days. These results represent a single experiment.

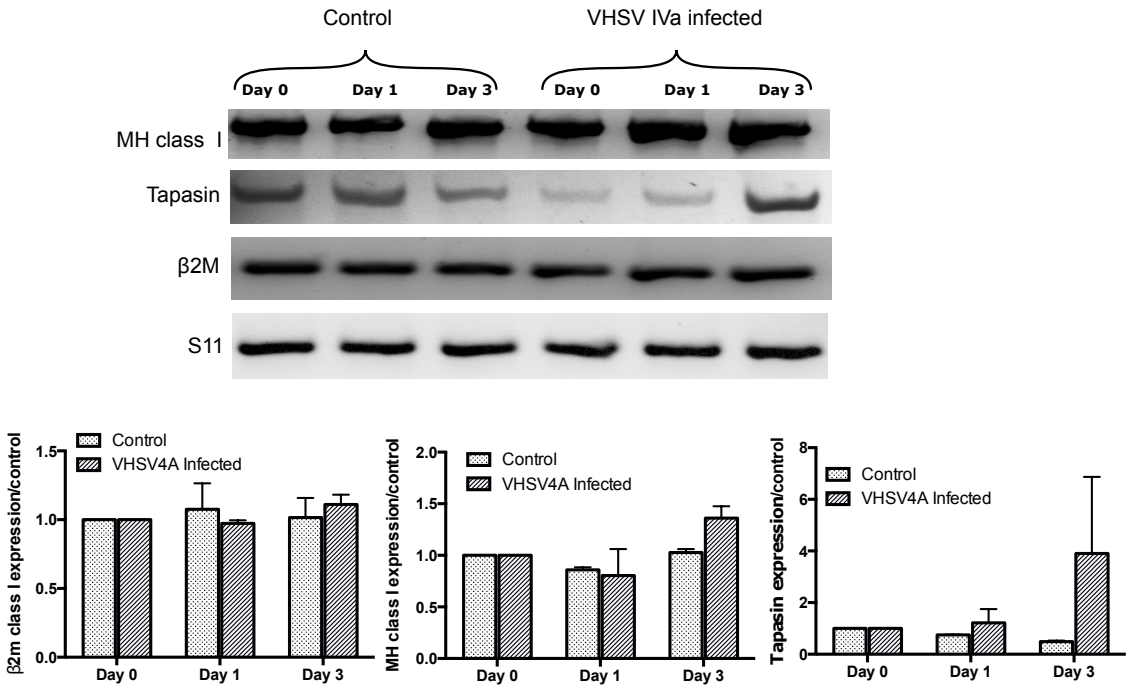

Figure 5.3.5: **RT PCR analysis of MH class β2m, tapasin expression in RTS11 after three days of infection with VHSV IVa.**

Transcripts expression levels of MH class I, β2m, tapasin in RTS11 after three days of infection with VHSV IVa. Densitometry analysis for MH class I heavy chain β2m and tapasin was conducted using Image J software. Vertical bars represent the standard error. These results are representative of two separate experiments. Ribosomal protein S11 amplicons were used as an internal standard control.

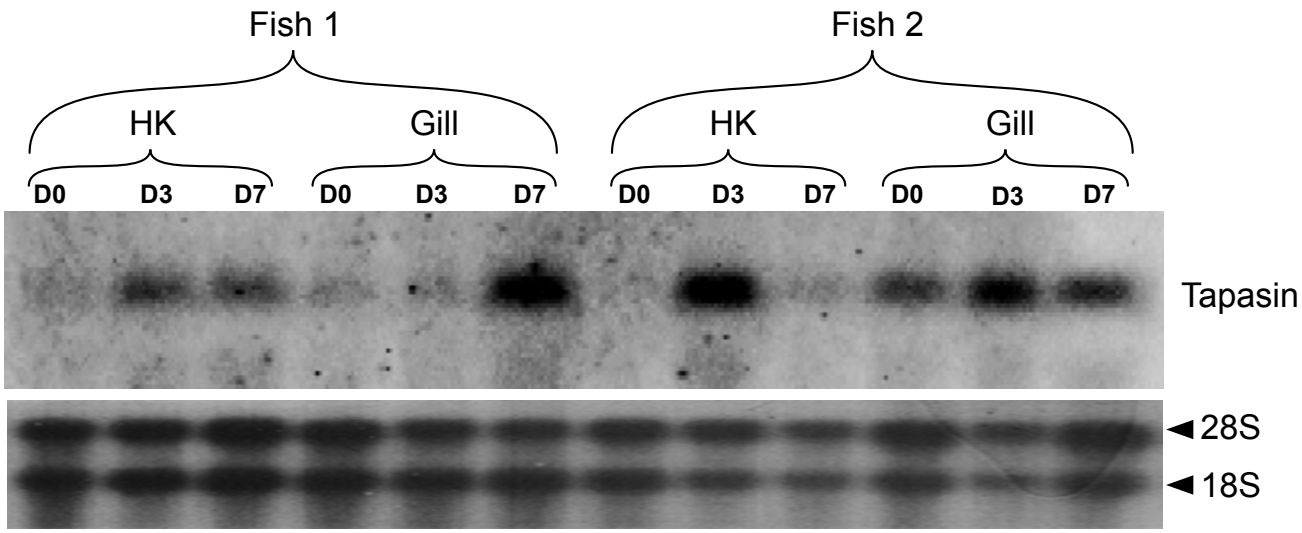

Figure 5.3.6: **Northern blot analysis of tapasin expression upon *in vivo* infection of rainbow trout with VHSV IVb.**

Expression of tapasin transcript in rainbow trout head kidney and gill tissues upon infection with VHSV IVb on day 0 (D0), day 3 (day 3) and day 7 (D7). Day 0 samples were obtained from a negative control fish. The position of 28S and 18S ribosomal bands are shown to indicate the RNA integrity and loading levels.

5.3.3 MH class I surface expression in VHSV IVa infected RTS11 at 14 degrees

In order to determine the MH class I surface expression, cultures of RTS11 were fixed with 0.25% paraformaldehyde to reduce the penetration of the antibody into the cells and allows the detection of MH class I mainly on the cell surface. As shown in Figure 5.3.7, MH class I surface expression was more pronounced in RTS11 infected cells than in the non-infected seven days post infection.

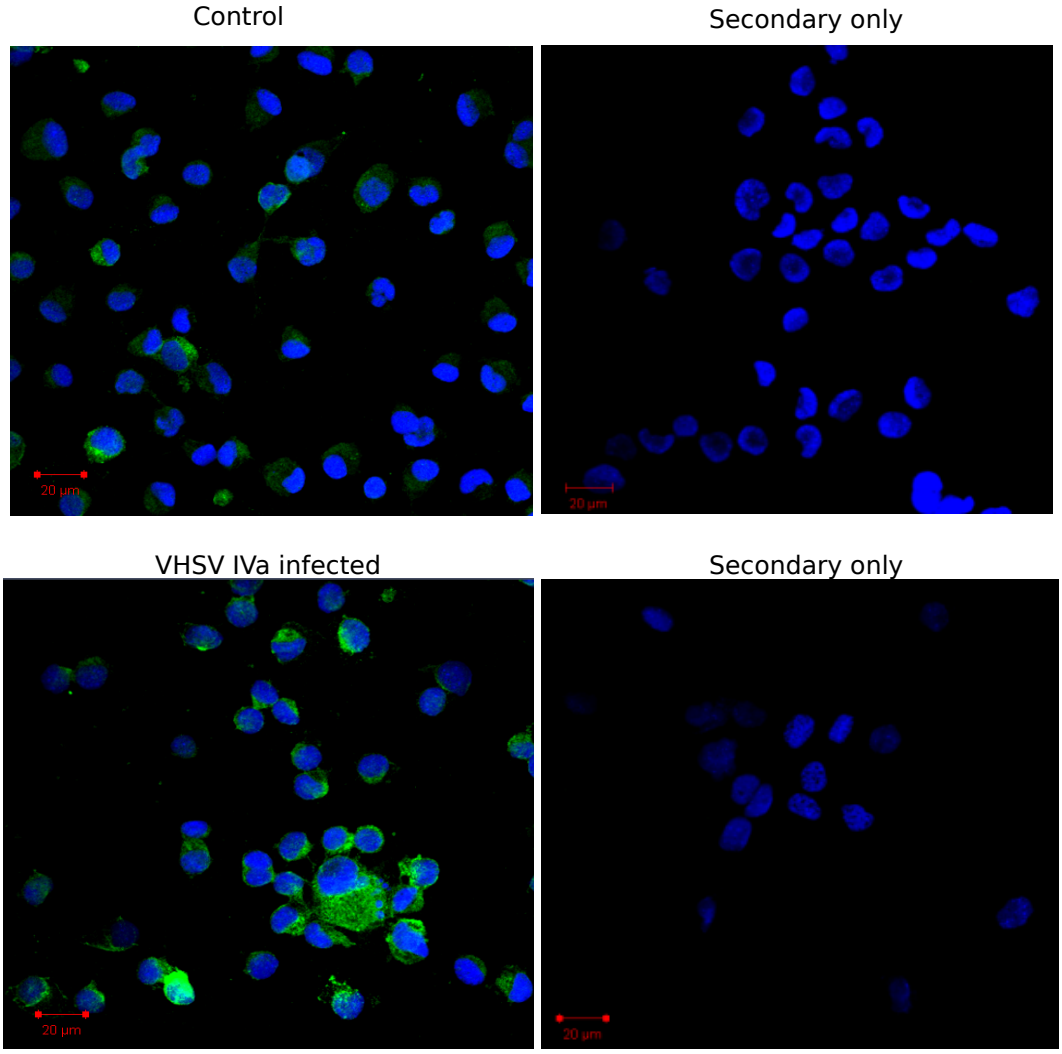

Figure 5.3.7: **Immunofluorescence detection of MH class I heavy chain on the cell surface of RTS11 after seven days of infection with VHSV IVa at 14 degrees**
Detection of MH class I heavy chain on the cell surface of RTS11 using a rabbit polyclonal antibody and secondary antibody conjugated to Alexafluor 488. Fluoroshield containing DAPI was used for nuclear staining. The negative control was stained with secondary antibody alone.

In addition, a small population of macrophages with a flatter morphology were observed and they were often associated with higher levels of MH class I surface staining, possibly indicating cellular differentiation of monocyte-like cells into differentiated APC-like macrophages in response to viral antigens. Moreover, staining of cellular processes was more readily detected in the infected cells perhaps pointing to altered dynamics in the cytoskeleton structure in these cells. These results support the cellular upregulation of the class I molecule at day 7 post infection noted above and further demonstrate that more class I molecules reach the cell surface, suggesting active antigen presentation by RTS11 cells.

5.3.4 β2m secretion into conditioned media of VHSV IVa infected RTS11

During normal internalization of the MHC class I molecule, β2m can be released at low levels into the extracellular fluid and can be detected in various body fluids such as: urine, plasma and saliva [213, 214, 215]. To investigate the regulation of β2m in RTS11 cells, conditioned media of RTS11 cells were collected to monitor the secretion of β2m under normal and viral induced conditions. As shown in Figure 5.3.8, β2m secretion was detected in RTS11 conditioned media and increased over time during normal conditions, however when levels of secreted β2m were measured in the conditioned media of infected cells, a higher turnover rate was observed which peaked up to 2 fold compared to the control cells 14 days post infection. Cell numbers did not vary significantly between control (non-infected) and infected cultures (data not shown), which indicates that the increased secretion levels detected in the infected cultures is not due to differences in cell numbers, but to increased MH class I antigen presentation pathway activity.

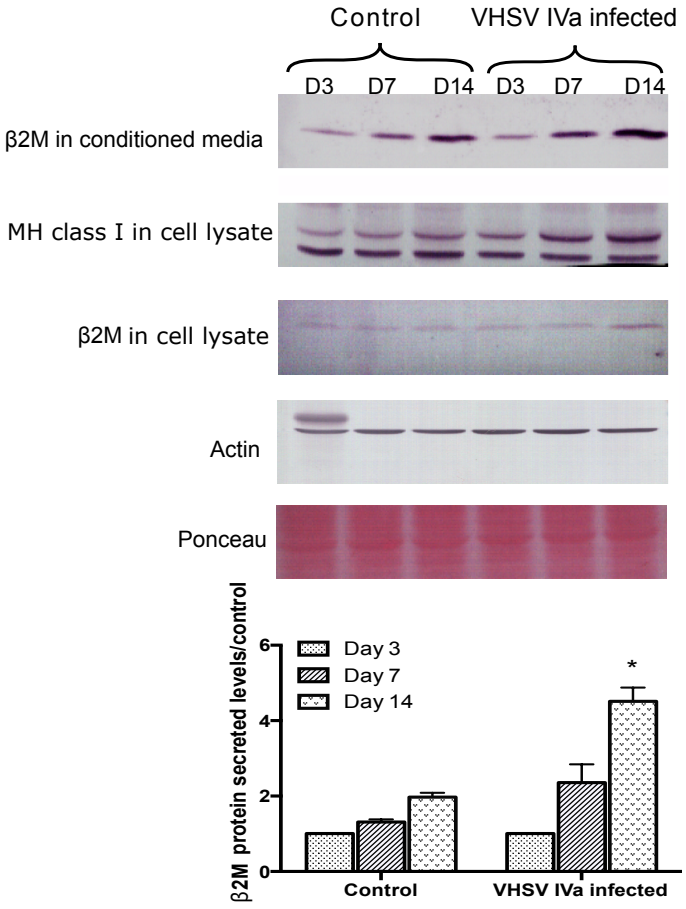

Figure 5.3.8: **Western blot analysis showing protein levels of β2m in conditioned media and levels of MH class I and β2m in whole cell lysates of control and VHSV IVa infected cells.** RTS11 cultures were infected with VHSV IVa for 14 days. Levels of β2m protein were detected in whole cell lysates and in conditioned media. Densitometry analysis was conducted using Image J software. Statistical analysis was performed using one-way ANOVA and Tukeys *post hoc* test. Significant differences between the control and treatment groups are represented by * ($P<0.05$) Vertical bars represent the standard error. These results are representative of three separate experiments.

5.3.5 MH class I expression in VHSV IVa infected RTS11 at 2 degrees

As fish susceptibility to viral infection can be dependent on temperature changes in their environment [216, 217], the regulation of the MH class I molecule under temperature dependent conditions was further investigated during VHSV infection.

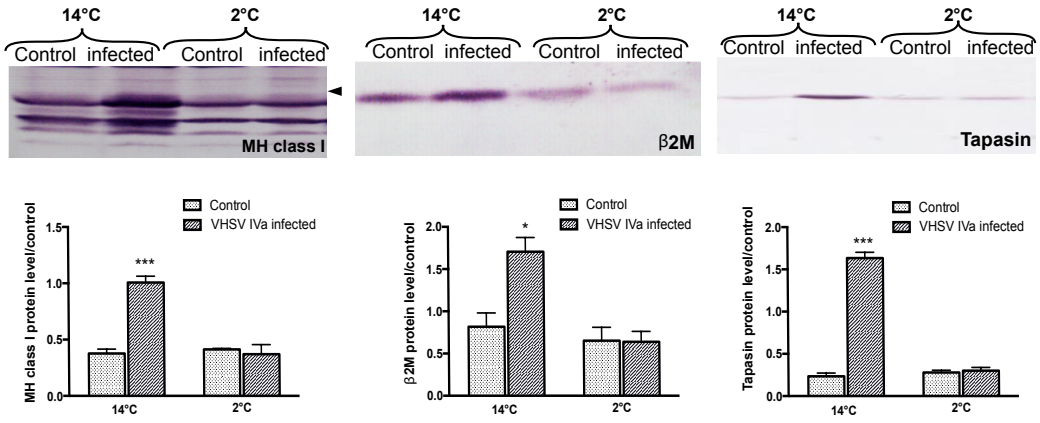

Figure 5.3.9: **Western blot analysis showing protein levels in whole cell lysates of MH class I heavy chain, β2m and tapasin in RTS11 upon infection for 9 days with VHSV IVa at 14 or 2 degrees.**
MH class I heavy chain, β2m and tapasin protein levels in RTS11 upon infection for 9 days with VHSV IVa at 14 or 2 degrees. The arrowhead represents the position of the glycosylated MH class I band. Densitometry analysis was conducted using Image J software. Statistical analysis was performed using one-way ANOVA and Tukeys *post hoc* test. Significance differences between the control and treatment groups is represented by * * ($P<0.05$) or *** ($P<0.001$). Vertical bars represent the standard error. These results are representative of three separate experiments.

As shown in Figure 5.3.9, the expression of cellular MH class I heavy chain, β2m and tapasin remained unchanged at 2°C under normal and viral infected conditions.

In contrast, a significant increase in expression was observed for all proteins when infection preceded at 14°C (Figure 5.3.9). Strikingly, when β2m levels were detected in conditioned media (Figure 5.3.10), a significant decrease in β2m accumulation was detected at 2°C relatively to cultures maintained at 14°C. Importantly, the decrease in β2m was also observed during infection with VHSV IVa, which suggests that MH class I antigen presentation and surface expression can be significantly reduced, which can compromise the cellular immunity against VHSV at this temperature.

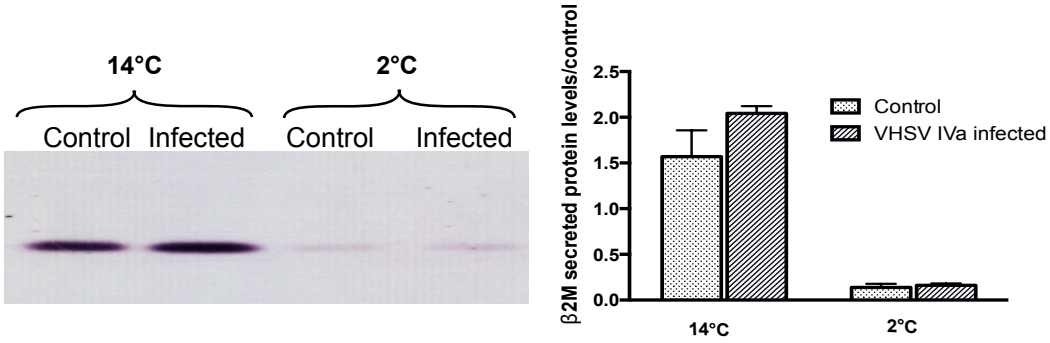

Figure 5.3.10: **Western blot analysis showing β2m secretion in RTS11 upon infection for 9 days with VHSV IVa at 14°C or 2°C.**

β2m secretory levels in RTS11 upon infection for 9 days with VHSV IVa at 14 or 2 degrees. Densitometry analysis was conducted using Image J software. Vertical bars represent the standard error. These results are representative of three separate experiments.

5.4 Discussion

In this study, the regulation of MH class I and its associated chaperones was examined during an anti-viral response to VHSV IVa at 14°C and 2°C. Unlike other VHSV IVa-permissible cell lines such as RTgill-W1 from trout gill epithelium, the failure of VHSV IVa to induce cytopathic effects in RTS11 [207] allows the examination and measurement of prolonged host MH class I expression in response to the virus. It has been recently demonstrated that VHSV IVa can produce viral transcripts in RTS11 cells, however this cell line could not support the production of newly synthesized virions [207]. These finding raise the interesting question whether MH class I molecules can be induced during infection of VHSV IVa when low levels of interferon transcripts are being produced [207] and when viral peptide supply in the cytosol is limited.

5.4.1 Induction of the MH class I but not ERp57 and calreticulin proteins by poly I:C

Poly I:C is known to be a potent inducer of the interferon response and therefore used as a tool to study mimic viral infection [218]. In mammalian dendritic cells has been shown to increase surface expression of MHC class I receptors bound to a peptide [219] and exerted maturation of dendritic cells both phenotypically and functionally by upregulation of molecules such as MHC class II, CD83 and CD86 [218]. Poly I:C treatment in fish

was previously shown to induce the transcription of the MH class I antigen presentation genes in the Atlantic salmon kidney (ASK) cell line three days post stimulus [99, 79] which further support the conserved induction of the MHC class I pathway by poly I:C, presumably through the interferon response. In contrast to the up regulation in MH class I, poly I:C treatment failed to induce increased expression of calreticulin and either at the transcriptional level [116].

In mammalian cells, calreticulin was shown to play a role in either inducing or inhibiting cellular proliferation depending on the cell type [220, 221]. Interestingly, a recent study, which used a proteomic approach to find novel TLR3 targets in mammalian cancer cell line, has demonstrated that poly I:C treatment differentially downregulated calreticulin almost 40% compared to the control, which appeared to have a positive effect on cellular proliferation [222]. It remains to be determined if calreticulin expression levels can be altered during poly I:C treatment using other trout cell lines, as calreticulin expression in RTS11 was shown not to be regulated also in response to other stimulants [117]. In addition, similarly to calreticulin, no change was observed for ERp57 at the protein level, following exposure to poly I:C, although, a short transcriptional induction was characterized for trout ERp57 [149].

5.4.2 Induction of the MH class I during VHSV IVa infection but not ERp57 and calreticulin proteins

In this study, the induction of MH class I at both the mRNA and protein level only occurred after day 3 post infection, whereas VHSV infection of the rainbow trout gonadal fibroblastic cell line RTG-2 demonstrated a more rapid induction of MH class I, which was upregulated at 8 h p.i. [213]. The differences in the kinetics of the induction in RTS11 versus RTG-2 could be the result of many possible factors such as: virus strain, viral titre, antibody sensitivities or might be due to distinct MH class I alleles present in these cell lines. Interestingly, RTgill, infected with VHSV IVa demonstrated no change in expression of MH class I three days p.i., although upregulation of MH class I did occur when exposed to poly I:C (data not shown). It is likely that the difference in the MH class I induction between RTgill and RTS11 may lie in the fact that RTgill exhibits CPE on day 4 in response to the virus and therefore induction of MH class I on day 3 can be compromised. Interestingly, albeit the lack of a foreign peptide supply in RTS11 [207], it appears that the presumed induction of the interferon response to VHSV IVa results in

persistent upregulation of the MH class I on the cell surface which can be a signal to alert other immune system cells to possible dsRNA intermediates in the cytosol.

A possible alert mechanism could be that MH class I on the cell surface can bind to exogenous viral peptides by exchanging them with self peptides or by direct binding to empty MH class I receptors. Interestingly, few studies using peptide feeding assays with mammalian cells have shown that MHC class I can bind *in vitro* to exogenously labeled peptides [223, 224, 225, 226, 227], however these assays were performed when peptide concentrations were much higher than normal physiological levels which implies that in live cells, exogenous peptide exchange on the cell surface is probably unlikely. Therefore, it can be alternatively suggested that exogenous VHSV peptides might may bind the MHC class I through the atypical mechanism of cross presentation which allow the access of extracellular peptides into the cytosol followed by presentation by MHC class I receptors [228]. This phenomenon was characterized in a subset of dendritic cells such as plasmacytoid DCs which possess the ability to take up exogenous antigens and present them to T cells [229]. Cross presentation can be advantageous in anti viral immunity when antigen presenting cells are not infected or when viral proteins are not produced.

Presumed uptake of extracellular viral antigens by RTS11 can help to initiate the immune response, albeit the lack of production on VHSV virions in the cytosol. In contrast to the upregulation of tapasin protein during VHSV infection, no change was observed at the protein level for calreticulin or ERp57, nevertheless, inducible expression for the latter has been previously seen following treatment with phytohemagglutinin and during exposure to calcium Ionophore [149]. Although, in this study both ERp57 and calreticulin failed to up regulate during VHSV infection, previous studies in mammalian cells have shown that both calreticulin and ERp57 can be regulated by viruses such as during Hepatitis B virus (HBV) and influenza infection which indicates their possible role in regulating viral replication in those species [230, 231]. Additional studies should be performed to elucidate the regulation of these chaperones in RTS11 during infection with other fish viruses to reveal if their expression could be regulated during viral infection.

5.4.3 Extracellular secretion of β2m during VHSV IVa infection

It has been shown that β2m can be secreted under normal and abnormal physiological conditions by various cell types of hematopoietic, epithelial and mesenchymal origin [232, 233]. This secretion was shown to be partly mediated by interferon gamma and interferon alpha

in different human cell lines [234, 235]. It is not yet clear whether the higher release of β2m during VHSV IVa infection is simply a by-product of enhanced MHC class I turnover on the cell membrane or that it has another functional immunological implication. Interestingly, some surprising alternate functions for this molecule were recently revealed. It has been shown in vitro that β2m can induce a chemotactic response in hematopoietic rat bone marrow cells [236, 237] and at high concentration, could alter cellular morphology and reduce the surface expression of the MHC class I in dendritic cells, which suggested its possible role as a regulator of the immune response [238]. It remains to be seen if similar regulatory effects of soluble β2m exist in the immune response of teleosts. It can be suggested that the enhanced secretion of β2m during VHSV IVa infection could be a result of a either poor stability of the MH class I receptor that might arrive on the cell surface without a foreign peptide or that this could be a possible mechanism to exchange the current peptide with other peptides in the surroundings by releasing β2m and subsequently independently engaging new β2m molecules and extracellular foreign peptides.

5.4.4 Protein levels of the MH class I and β2m during infection with VHSV IVa at 14 degrees and 2 degrees

A study examining the temperature effect on β2m expression in trout and Atlantic salmon showed that β2m protein levels were maintained at 2°C for for 10 days under non-stimulating conditions [99]. In addition, immunohistochemistry showed that the surface expression levels of β2m in peripheral blood leukocytes could persist at the cell surface at 2 degrees in unstimulated cells [99]. However, β2m levels in the media reflect MH class I receptor turnover and thus, it can be speculated that the trafficking and/or delayed transcription/translation of MH class I receptors are likely to be attenuated at low temperature. Thus, under these conditions, antigen presentation on the cell surface can be substantially slowed down, which will allow the virus to replicate freely without being eliminated by immune responses.

5.4.5 Summary

This study demonstrated the induction of the glycosylated MH class I protein in RTS11 cells following poly I:C treatment and during VHSV IVa infection. in RTS11 cells following poly I:C treatment and during VHSV IVa infection. In addition, tapasin expression

levels were shown to be unregulated during VHSV IVa infection, whereas no induction was observed for ERp57 or calreticulin. The induction of the MH class I, was found to be temperature sensitive which may partly contribute to compromised immune responses against this virus at this low temperature. Importantly, we demonstrate higher accumulation of β2m in the conditioned media of RTS11 during VHSV IVa infection, which needs to be further explored for its potential distinctive functions during the activation of the immune response.

Chapter 6

The teleost peptide loading complex

6.1 Introduction

The recognition of intracellular pathogens such as viruses by cytotoxic T cell lymphocytes (CD8+) is mediated through the MHC class I receptor which present foreign peptides on the cell surface that marks infected cells for destruction [20]. The MHC class I molecule is composed of three essential components: the polymorphic heavy chain, $\beta 2$ microglobulin and the foreign peptide which assemble together in endoplasmic reticulum. Lack of any of these three components results in poor stability and degradation of the MHC class I receptor. Therefore, a tightly regulated pathway has evolved in the endoplasmic reticulum (ER) which accommodates the unique folding requirements of the MHC class I receptor and loading it with a diverse pool of peptides [6, 239].

Under normal conditions, peptides to be loaded on the MHC class I are mainly generated by proteasomal cleavage of native proteins or from DRiPs derived from abberent transcription or translation [15]. During infection with intracellular pathogens, the peptides drived from those pathogens are degraded by a proteasome modified to produce MH specific peptides [14]. The trafficking of the peptides from the cytosol into the ER involves the transporter associated with antigen processing (TAP), a member of the ATP binding cassette family which preferentially delivers peptides of 8-16 amino acids [17]. Evidence for TAP function during antigen presentation came from studies using TAP deficient cells which display reduced levels of MHC class I on the cell surface and fail to present endogenous peptides to CD8+ cells [18]. In humans, the folding of the MHC class I receptor can be divided into two distinct steps: during early folding in the ER, the MHC class I

heavy chain associates with the lectin chaperone calnexin and also with the thiol-disulfide oxidoreductase, ERp57.

In humans, upon assembly with $\beta 2$ microglobulin, the MHC class I heavy chain is incorporated into the peptide loading complex (PLC) containing TAP, the lectin chaperone calreticulin and a disulphide linked conjugate of tapasin [37, 174] and ERp57 [6, 174, 8]. The first teleost major histocompatibility genes fragments were initially isolated from carp [60] and further research revealed a surprising genetic architecture in which the major histocompatibility genes are not linked as in other vertebrates and therefore they are to referred as MH genes [83, 84].

The teleost genes involved in antigen presentation such as TAP and the proteasome induced subunits LMPs (low molecular mass polypeptide) have been cloned, characterized and mostly mapped as being linked to MH class I classical genes [76, 85]. Later, tapasin and tapasin related genes were identified and were shown to be upregulated during *in vivo* viral infection [86, 122, 98]. In addition, genes encoding the molecular chaperones participating in the peptide loading complex have been identified and studied in several teleost fish [116, 149, 148]. To date, in fish, no studies have investigated the MH class I assembly in the ER, mainly due to the lack of available antibodies. Elucidating this pathway in fish is important for understanding of the immune response in fish, in particular mechanisms of immune surveillance, T cell activation and peptide selection during fish viral infection.

6.2 Material and methods

6.2.1 Fish Cell Lines

RTS11, a rainbow trout spleen monocyte/macrophage cell line [107] was maintained at 20°C in Leibovitzs L-15 medium (ThermoFisher Scientific, Nepean, ON) with 20% FBS containing 150 U/mL of penicillin and 150 mg/mL streptomycin in 75 cm^2 culture flasks. The EPC cell line derived from fathead minnow [209] was maintained in L–15 medium with 10% FBS in 75 cm^2 culture flasks at 26°C and sub-cultured every 7 days by trypsin (Lonza, Allendale, NJ). The following trout cell lines were grown in room temperature in L-15 with 10% FBS, RTmt from the testis and RTovarian fluid from the ovarian fluid (TK Vo and NC Bols). Long-term fin leukocyte-like cultures were grown *ex vivo* from fin explants from rainbow trout (TK Vo and NC Bols). These cells were maintained in L-15 with 30% FBS at 20°C.

6.2.2 VHSV IVa propagation and Infection of RTS11 cells for immunoprecipiation

North American group viral hemorrhagic septicemia virus IVa isolate (VHSV IVa) from Pacific herring [210] was routinely propagated on EPC cultures in L-15 medium with 2% FBS at 14°C. Once the confluent cell monolayer was completely destroyed, the virus-containing conditioned media was spun at $4500 \times g$ for 5 minutes at 4°C. The supernatants were filtered through 0.2 μm membranes (Pall Corporation, Mississauga, ON) and stored in aliquots at -80°C for later use. Viral titres were determined by $TCID_{50}$/mL assays as previously described [207]. For cell infection, approximately, 1×10^8 RTS11 cells in L-15 media supplemented with 2% FBS were challenged with 1×10^8 $TCID_{50}$/mL of VHSV IVa. The control (non-infected) flask contained the same number of cells without the virus and incubated for 7 days at 14°C. Cells were then collected and centrifuged at $500 \times g$ for 4 minutes at 4°C. Subsequently, cell pellets were incubated on ice-cold PBS containing 10 mM of methyl methanethiosulfonate (MMTS: ThermoFisher Scientific, Nepean, ON) for 3 minutes followed by quick centrifugation at $500 \times g$ to obtain cell pellets.

6.2.3 Immunoprecipiation

RTS11 and fin primary cultures (1×10^8) were collected and incubated for 2 minutes with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4; pH 7.4) containing 10 mM of MMTS (ThermoFisher Scientific, Nepean, ON). Subsequently, cells were centrifuged at $500 \times g$ for 3 minutes and lysed with 1% digitonin (Sigma Aldrich, St. Louis, MO) containing 10 mM MMTS, 50mM of Tris-HCl, 150 mM of NaCl supplemented with 1X protease inhibitors (Roche, Mannheim, Germany). Cells were rotated at at 4°C for 30 minutes followed by centrifugation at 10,000 rpm for 30 minutes to remove cellular debris. Immunoprecipitation was performed using either 1:50 affinity purified Tapasin antibody, pre immune tapasin rabbit serum 1:200, anti-MH antibody [79] or 1:200 of anti TAP1 antibody (SAB2102370: Sigma Aldrich, St. Louis, MO). Antibodies were rotatated with lystates overnight at 4°C, followed by the addition of 50 μL of protein A agarose beads (Sigma Aldrich, St. Louis, MO) for 1 h. The beads were spun down by centrifugation at 3000 rpm and subsequently washed three times with 500 μL of 0.1% of digitonin lysis buffer. Immunoprecipitated elutions were separated on 10% SDS page gel, transferred to a nitrocellulose membrane and probed with either anti MH class I (1:400) antibody, anti-tapasin antibody, anti-human TAP1 and anti-human calnexin (GTX101676) antibody

for one hour. Detection was performed using anti-rabbit IgG VeriBlot (HRP) diluted with 1:800 of skim milk and clarity ^{TM}(Roche, Mannheim, Germany) according to manufacturers instructions.

6.2.4 Western blotting

For the detection of the interaction between ERp57 and tapasin, non-stimulated RTS11 cells (2×10^6) were collected and incubated for 2 minutes with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4; pH 7.4) containing 10 mM of MMTS (ThermoFisher Scientific, Nepean, ON). Subsequently, cells were lyzed for 30 min with 1% digitonin (Sigma Aldrich, St. Louis, MO) containing 10 mM of MMTS, 50mM of Tris-HCl, 150 mM of NaCl supplemented with 1x protease inhibitors (Roche, Mannheim, Germany). Supernatants were collected after centrifugation at 13,000 rpm for 20 min. Protein samples were prepared under reducing with β mercaptoethanol or non-reducing conditions, without β mercaptoethanol) and loaded onto 10% SDS page gel and probed with either anti-ERp57 antibody (1:1000) or anti-tapasin antibody (1:200). Detection was performed using anti-rabbit IgG VeriBlot (HRP) diluted with 1:800 of skim milk and clarity ^{TM} (Roche, Mannheim, Germany) according to manufacturers instructions. For detection of tapasin protein in RTtestis and RTovarian fluid, 2×10^6 cells were lysed in 1% NP-40 lysis buffer containing 150 mM NaCl and 50 mM Tris [pH 8.0] supplemented with 1X of protease inhibitor cocktail (Roche, Mannheim, Germany). Cell lysates were separated on a 12% acrylamide gel and membranes (Bio-Rad, Mississauga, ON) goat anti-Rabbit alkaline phosphatase antibody (1:30,000) (Sigma Aldrich, St. Louis, MO). Detection used NBT/BCIP (Roche, Mannheim, Germany) according to the manufacturers instruction.

6.3 Results

6.3.1 Tapasin associates with the MH class I under normal and viral induced conditions

Trout tapasin protein was previously shown to be unregulated during VHSV IVa infection in RTS11 cells (Figure 5.3.4), which supported its conserved regulation during viral infection in a manner similar to mammals. However no evidence exists regarding its possible interaction with MH class I receptors. As shown in Figure 6.3.1A the association of MH

class I with tapasin was detected as the MH class I antibody detected MH class I receptor in the tapasin immunoprecipitates ofboth in control and infected cells.

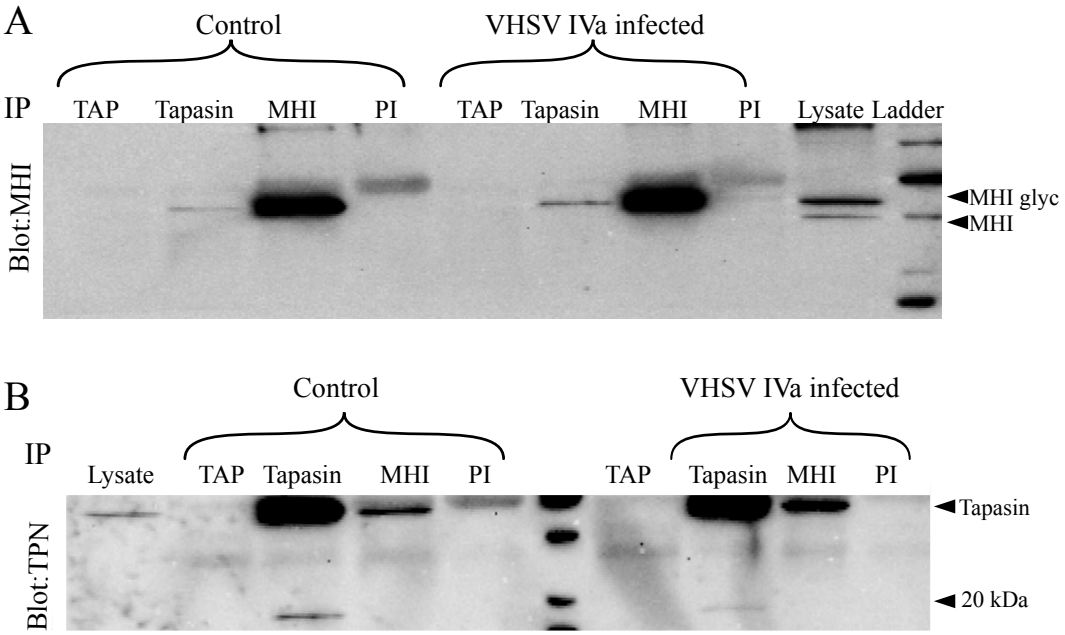

Figure 6.3.1: **Detection of the interaction between MH class I heavy chain with tapasin by co-immunprecipitation.**
RTS11 cells were infected with VHSV IVa for seven days followed by extraction in 1% digitonin lysis buffer. Immunoprecipitations were performed in cell lysates obtained from control and VHSV IVa infected cells with either tapasin antibody (tapasin), TAP antibody (TAP), MH class I antibody (MHI) or pre immune serum (PI). Blots were probed with either the MH class I antibody (A) or with tapasin antibody (B). The position of MH class I glycosylated band (MHI glyc) is represented in (A) with a arrowhead on the right margin.

This association was identified between tapasin and the upper MH class I band, previously shown to be the glycosylated form in RTS11 cells (Sever et al., unpublished results). In addition, when the blot was probed with tapasin antibody, the same interaction was observed as tapasin can be seen in the anti-MH class I immunoprecipitates, further supporting the reciprocal interaction of tapasin and the MH class I (Figure 6.3.1B). Furthermore, a 20 kDa protein was pulled down in tapasin immunoprecipitates and was detected by tapasin antibody, in addition to the predicted 48 kDa protein (Figure 6.3.1B).

This interaction of tapasin with the glycosylated form of the MH class I was also detected in long-term fin leukocyte-like cells, that contain many cells with characteristics of dendritic cells (TK Vo, and NC Bols) which further implies that this interaction is conserved in antigen presenting cells *in vivo* (Figure 6.3.2).

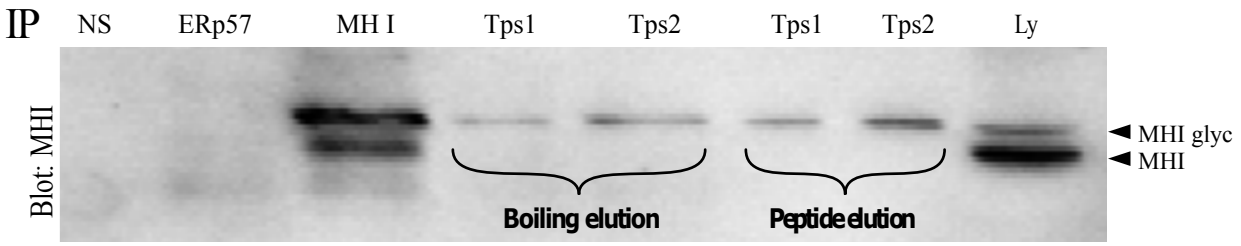

Figure 6.3.2: **Detection of tapasin and MH class I association in long-term fin leukocyte-like cells.**
A primary culture of fin leukocyte-like cells was extracted with 1% digitonin lysis buffer followed by immunoprecipitation with non-specific serum (NS), ERp57, tapasin serum (Tps1), affinity purified tapasin (Tps2) and MH class I heavy chain. Elutions were obtained using a synthetic tapasin peptide, followed by a second boiling elution. Immunoprecipitates were separated on 10% SDS PAGE gel and blotted with MH class I heavy chain antibody. The MH class I heavy chain bands are shown in the cell lysates (Ly). The position of MH class I glycosylated band (MHI glyc) is indicated with a arrowhead on the right margin.

6.3.2 TAP association with tapasin and MH class I under normal and viral infecting conditions

Tapasin in mammals is known to associate with TAP through its transmembrane domain which facilitates a structural bridge which brings TAP closer to the MHC class I [6]. In order to find if the tapasin: TAP interaction is conserved in fish, RTS11 cells were immunoprecipitated with human anti TAP1 antibody which was developed against a 50 aa peptide which shares 61% identity with the trout TAP1 protein. In Figure 6.3.3 trout TAP is detected in RTS11 cell lysates as a 75 kDa protein and its association with tapasin and MH class I was identified both in control and VHSV IVa infected cells.

Although, in Figure 6.3.3A immunoprecipitation with TAP antibody failed to detect any presence of TAP protein, when the concentration of TAP antibody for immunoprecipitation was doubled (Figure 6.3.3B), TAP protein could be identified in the pull down in parallel with its interaction with tapasin, further supporting their reciprocal interaction.

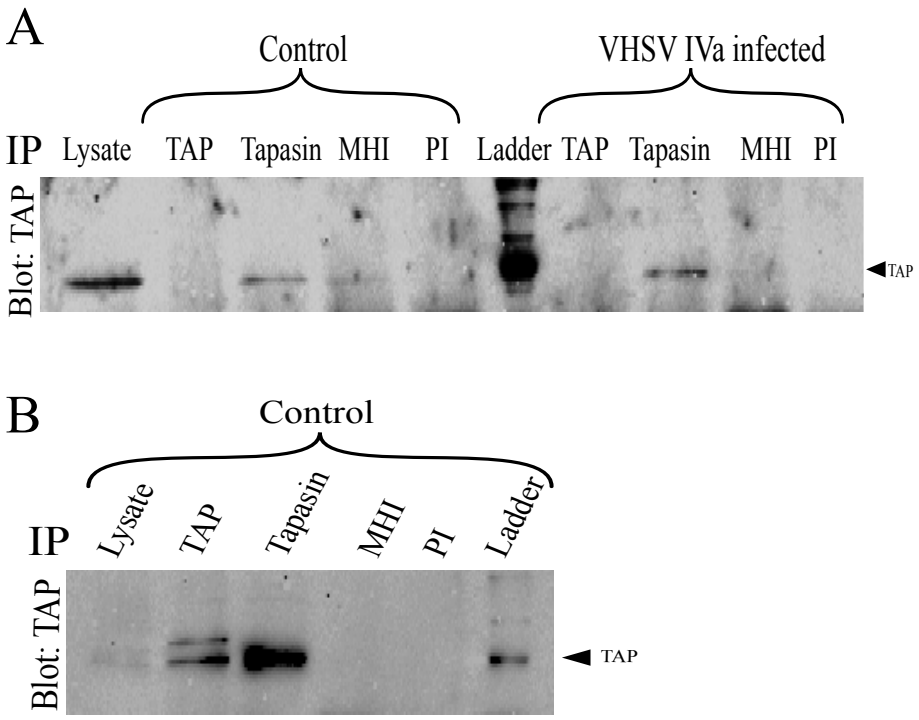

Figure 6.3.3: **Detection of the interaction between tapasin and TAP in RTS11 cells by co-immunoprecipitation.**
RTS11 cells were infected with VHSV-IVa for seven days followed by extraction with 1% digitonin lysis buffer. Immunoprecipitation was performed with either tapasin antibody (tapasin), TAP antibody (TAP), MH class I antibody (MHI) or pre immune serum (PI) and blots were probed with TAP antibody. The upper panel and lower panel represent two separate experiments.

6.3.3 Calnexin association with the MH class I and ERp57

Calnexin was first identified in mammals as a protein that associated with the MHC class I heavy chain [28, 126, 29] and is also known to associate through its proline rich domain with ERp57 [136]. Trout MH class I protein is glycosylated in rainbow trout [117] which suggested its possible interaction with lectin chaperones such as calnexin. Previous finding demonstrated that calnexin protein is detected in RTS11 as two protein forms of 120 kDa and 100 kDa respectively [240]. When RTS11 cells were infected with VHSV IVa, the association of both calnexin forms with the MH class I was detected (Figure 6.3.4A).

In addition, the 100 kDa band of calnexin was found to be present in ERp57 immunoprecipitates which implies their possible interaction. Under normal conditions, calnexin association with both the glycosylated and non-glycosylated form of MH class I was observed (Figure 6.3.4B). In contrast, calreticulin only interacted with the glycolsylated form of MH class I .

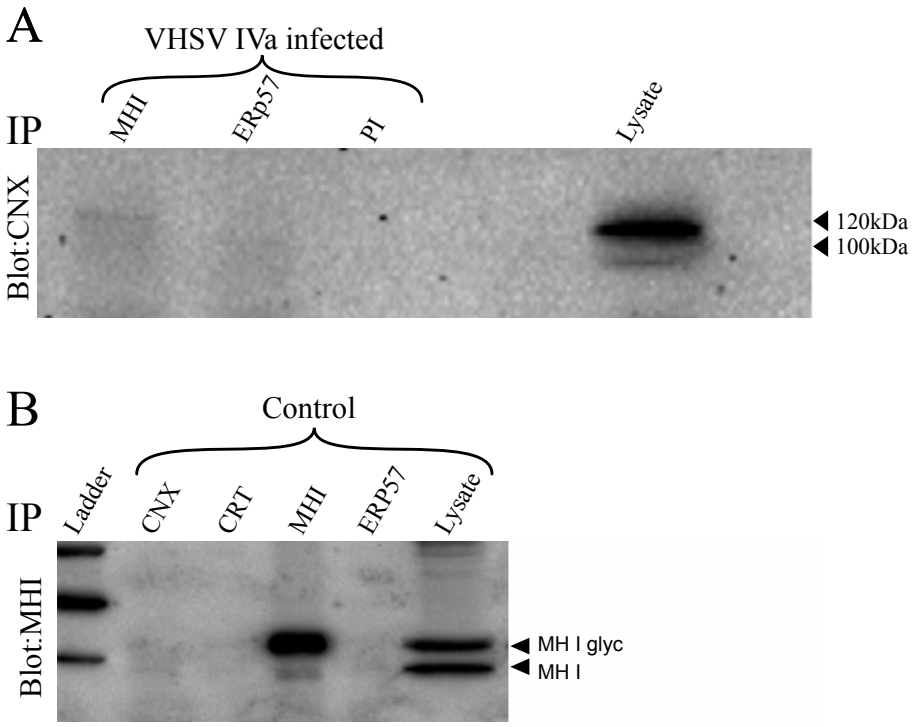

Figure 6.3.4: **Detection of the interaction between the of calnexin and calreticulin with the MH class I heavy chain protein in RTS11 cell line by co-immunoprecipitation.**
A) RTS11 cells were infected with VHSV IVa for seven days and cell lysates were immunoprecipitated with the antibodies ERp57, MH class I (MHI), and pre immune serum (PI), followed by a western blotting with calnexin antibody (CNX). The position of the two calnexin protein forms is marked with arrowheads on the right margin. B) Non-stimulated RTS11 cell lysates were immunoprecipitated with either ERp57, MH class I (MHI), tapasin, calreticulin (CRT), calnexin (CNX) and pre immune serum (PI), followed by a western blotting with MH class I antibody. The position of the glycosylated MH class I (MHI glyc) is marked with an arrowhead on the left margin.

6.3.4 Tapasin association with ERp57

To elucidate the possible interaction of ERp57 with tapasin, RTS11 cells were pretreated prior to the detergent extraction with methyl methanethiosulfonate (MMTS), which protects native disulfide bonds during detergent extraction [40, 37] and therefore preserve the disulfide linked interactions which can be easily abolished during cell lysis. When cell lysates from RTS11 cells were prepared under non reducing conditions, a 110 kDa heterodimer of tapasin and ERp57 was detected, similar to the conjugate seen in mammals (Figure 6.3.5). In contrast, under reducing conditions, the heterodimer expression was much less abundant in the tapasin blot and not seen at all in the ERp57 blot which implies

that this protein interaction is maintained via disulfide bonds. Interestingly, an additional 75 kDa band was detected both by tapasin and ERp57 antibodies under non-reducing conditions. This finding implies that a possible 20 kDa protein that contains the epitope of the tapasin antibody may associate with the 55 kDa ERp57 via a disulfide bond.

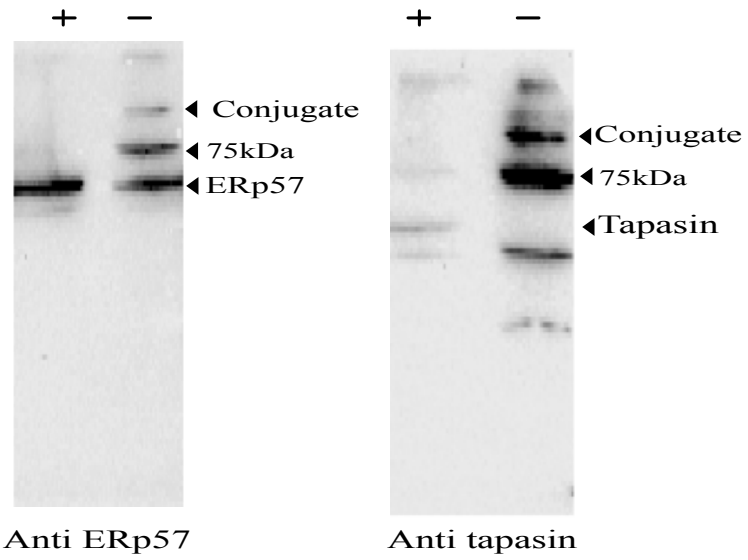

Figure 6.3.5: **Detection of a possible ERp57/tapasin disulfide linked heterodimer.** Western blot analysis of RTS11 pretreated with MMTS under reducing conditions with β mercaptoethanol (+) and under non reducing conditions without β mercaptoethanol (-). Blots were detected with either ERp57 or with tapasin antibody. Arrows indicate the putative ERp57-tapasin conjugate, the free ERp57–tapasin proteins and the novel 75 kDa conjugate.

6.3.5 Tapasin protein expression in trout reproductive cell lines

Trout tapasin protein is mainly detected as a 48 kDa protein which corresponds with its predicted molecular weight (Figure 4.3.1). However, an additional 20 kDa band was detected by tapasin antibody in trout cell lines and tissues (Figure 4.3.1) and was the only band detected in RTmt and in RTovarian fluid (Figure 6.3.6) suggesting a possible truncated version for tapasin protein which contains an intact c-terminal domain which tapasin antibody recognizes (Figure 6.3.1).

Figure 6.3.6: **Tapasin protein distribution in rainbow trout cell lines under non-stimulated conditions.**
Western blot analysis of tapasin protein in different trout cell lines demonstrating the detection of two distinct bands in rainbow trout cell lines: the predicted 48 kDa and an additional 20 kDa band in cell lines derived from the testis (RTmt) and the ovarian fluid (RTovarin fluid). The position of the molecular mass markers are shown in the right margin.

6.4 Discussion

In this work, the potential protein:protein interactions which may be involved in loading of peptides on the MH class I in teleost fish were examined. Although, indirect evidence suggests that antigen presentation does occur in teleost fish [241, 90, 213], the molecular mechanisms involved in peptide loading are still unknown. The spleen macrophage like cell line (RTS11) was used as our model since the trout proteins such as: calreticulin [117], ERp57 [149], and calnexin [240] were previously studied in this cell line. Importantly, both MH class I and class II proteins are abundantly expressed in RTS11 [79] which suggests that these cells are capable of presenting peptides and thus expresses high levels of proteins involved peptide loading pathways.

6.4.1 Interaction of tapasin with the MH class I

Tapasin is a key component in the peptide loading complex in mammals, acting as a bridge between MHC class I and TAP and has a dual role in stabilizing TAP [49] and in selecting high affinity peptides [242, 51]. The interaction of MHC class I heavy chain and tapasin is not fully understood due to their low affinity [6]. Nevertheless, studies using tapasin mutants showed that both of its immunoglobulin (Ig) luminal domains can interact with the MHC class I [243, 244, 45] and that deletion of the first 50 aa in the N terminal domain of human tapasin diminished the binding of tapasin to MH class I, ERp57 and calreticulin [245].

In this study, the association of trout tapasin with the trout MH class I molecule has been identified which suggests that tapasin can act as a MH class I chaperone and support the MH class I assembly in fish. Interestingly, tapasin protein is detected in both human and mice as a single 48 kDa band, whereas in trout cell lines tapasin is detected as the predicted 48 kDa protein with an additional 20 kDa band, which is expressed at different levels in different cell lines. In RTS11 cells both bands are expressed, however in the reproductive tissue derived cell lines, RTmt and RTovarian fluid, only the 20 kDa band is present. Thus the 75 kDa band detected in Figure 6.4.5 could be composed of the full length ERp57 protein and this 20 kDa truncated tapasin form. This finding raises an interesting possibility for the trout truncated tapasin form. As anti-trout tapasin recognizes the c terminal domain of tapasin, it could be speculated that the 20 kDa protein contains an intact cytoplasmic tail which could interact with TAP and also since this truncated form can associate with ERp57, it has enough of the N-terminus to contain the cysteine used for that disulphide linkage. Thus in order to be truncated, it must be missing domains in the middle, such as the Ig domains which interact with the MH class I and thus could be an alternative splicing variant.

Evidence for tapasin alternative splice variants was originally identified in the human B-lymphoblastoid cell line .220, which exhibits defective antigen presentation and as a result exhibits reduced MHC class I levels on the cell surface [175, 246]. This phenotype could be restored when a full length cDNA sequence of tapasin was transfected into .220 cells [42] but also when cells were transfected with a soluble version of tapasin which lacks its transmembrane and cytoslic domains [49]. Further study revealed that the genetic basis for the phenotype in .220 cells was a splicing of exon 2 which results in a truncation of the signal peptide and absence of 49 aa of the N terminal domain [247]. Additionally a splice variant of tapasin lacking exon 3 was recently identified in a human cancer cell line which demonstrated that in spite the intact binding of tapasin to TAP, the MHC class I surface expression was still impaired when cDNA encoding the splice variant was transfected into tapasin deficient cells [194]. Based on these findings, it is possible that the trout 20 kDa protein is an alternative spliced version of tapasin which could compete with the full length protein for binding to TAP, yet does not bring MH class I receptor into the complex. It could be speculated that expression of both tapasin versions allows regulation of antigen presentation at important immune system sites such as the head kidney and spleen during normal conditions. In contrast, in sites such as testis and ovaries where restricted immune responses exist, expression of high levels of the truncated tapasin can be desirable (Figure 6.4.1).

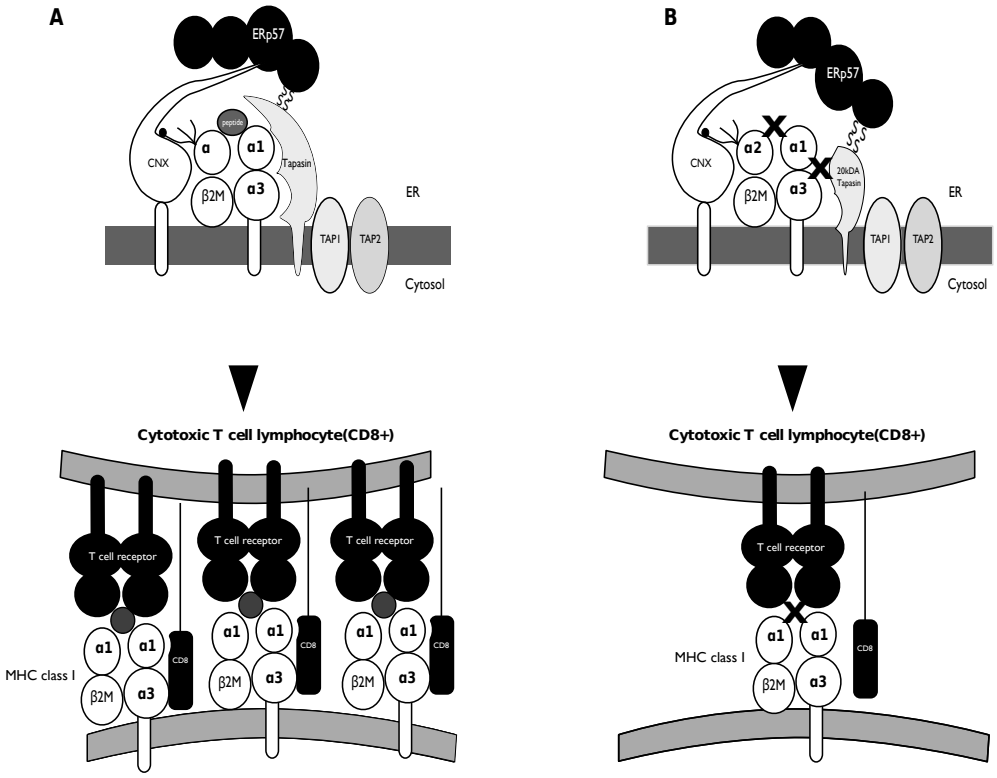

Figure 6.4.1: **A proposed model for the possible role of the 20 kDa protein in the MH class I assembly during viral infections**

(A) During viral infection, the expression of the full length tapasin is enhanced, which allows tapasin to bind to the MH class I and outcompetes the truncated 20 kDa protein in binding to TAP which facilitates MH class I assembly and antigen presentation. In contrast, in immune privileged sites, or in unstimulated tissues (B) such as testis and ovaries, the protein levels of the truncated tapasin version is higher than the full length which impairs the recruitment of the MH class I to tapasin and therefore results in poor maturation of the MH class I and compromised antigen presentation.

As previous findings showed that only the full length tapasin is unregulated during viral infection (Figure 4.3.6A) , it is possible that the full length tapasin outcompetes the truncated form in binding to TAP allowing a more MH class I to enter the peptide loading complex and thus more rapid and efficient antigen presentation in response to intracellular infection. tapasin deficient cells [194]. Based on these findings, it is possible that the 20 kDa protein is an alternative spliced version of tapasin which could compete with the full length protein for binding to TAP. It could be speculated that expression of both tapasin version allows regulated antigen presentation in important immune system sites such as the head kidney and spleen during normal conditions. In contrast, in sites such as testis and ovaries where restricted immune response exist, expression of high levels of the truncated

tapasin can be desirable (Figure 6.4.1). As previous finding showed that only the full length tapasin is unregulated during viral infection (Figure 4.3.6A), it is possible that under these conditions the full length tapasin outcompetes the truncated form in binding to TAP which allow a more rapid and efficient antigen presentation in response to intracellular infection.

6.4.2 Tapasin association with TAP

A transmembrane domain leucine motif known to be involved with TAP binding in mammals [248] is absent from the trout sequence, however three leucine residues do appear in the transmembrane domain of trout tapasin that could putatively interact with TAP [86]. Additional studies showed that other residues in tapasin transmembrane domain might also be involved in binding TAP, such as the charged lysine residue in position 408 in the middle of the transmembrane region [249] which is conserved in zebra fish and rainbow trout tapasin sequences. Therefore, the interaction of trout tapasin with TAP could be mediated through tapasins transmembrane domain similarly to mammal.

6.4.3 Tapasin association with ERp57

Interestingly, trout tapasin contains amino acids substitutions which may influence its putative protein interactions with ERp57 and TAP. In all teleost tapasins studied to date the conserved Cys95, known to recruit ERp57, is substituted with serine, a feature surprisingly also conserved in birds [250]. Nevertheless, another upstream cysteine residue in position 57 which is found in only teleosts could possibly replace Cys95 in binding ERp57. The ERp57-tapasin interaction in mammals is detected as a 110 kDa disulfide linked hetrodimer on a western blot under non reduced conditions [40, 37]. The data presented in Figure 6.3.5 indicate a possible heterodimer conjugate formation with a relative similar molecular weight in trout. However, unlike mammals, an additional 75 kDa band is detected by both ERp57 and tapasin antibody which further support the possibility of 20 kDa tapasin version that interacts with ERp57 and plays a role in antigen presentation.

6.4.4 Calnexin and calreticulin association with the MH class I

Calnexin and calreticulin are lectin chaperons which bind N-linked glycans in the ER and contain a conserved docking site for ERp57 [136] in the P domain, which was also shown to be conserved in fish [116, 148]. In mice, calnexin was shown to associate with the nascent

MHC class I receptor which consists the assembled MHC class I heavy chain and β2m. However, human calnexin was shown to bind the MHC class I heavy chain alone [6, 8]. This report show the interaction of calnexin and with ERp57 and the MH class I during VHSV IVa infection and demonstrates that during normal conditions, calreticulin can associate with the glycosylated a form of the MH class I, whereas calnexin appears to associate only with both the glycosylated and the non-glycosylated forms.

6.4.5 Summary

In this work, the conserved association of the MH class I with calnexin, calreticulin and tapasin was identified, along with the interaction of tapasin with TAP, and ERp57 similar to mammals. This suggests that a PLC very similar to mammalian antigen presentation pathways exists in fish. However, unlike mammals another 20 kDa tapsin protein was identified, which is suggested here to be a truncated version that may have a regulatory role in antigen presentation in teleosts. Although, many protein interactions were identified in this report, their exact contributions to peptide loading are far from being understood. Future work, will aim to elucidate the contribution of such interactions for antigen presentation and T cell response and also will pursue the identity and fucntion of the 20 kDa band.

Chapter 7

Discussion and future aims

7.1 ERp57

7.1.1 Functional charectrization

ERp57 is a protein disulfide isomerase which in human resides and functions mainly in the ER. The full length cDNA sequence of rainbow trout ERp57 was characterized for the first time in this thesis and found to be widely expressed in trout tissues. Southern blotting revealed that there are ERp57 in at least two copies present in the trout genome. The putative protein had 75% sequence identity with its mammalian homologues and is ubiquitously expressed in trout as a 57 kDa protein.

Interestingly, unlike its mammalian homologues, trout ERp57 contains neither an ER retention motif nor a nuclear localization signal both of which are conserved in mammals, chicken and zebrafish. This suggests that trout ERp57 may have a function outside of the ER in trout. Surprisingly, a database search (GeneBank TSA) revealed another coding sequence for ERp57 in trout that encodes a ERp57 protein with a putative NLS, suggesting its a reason for differential localization and perhaps alternate function. Using specific monoclonal antibodies against the two different trout ERp57 protein versions could help elucidate their sub-cellular localization as well as their unique regulation in trout. A recent study has demonstrated that ERp57 assists in the translocation of calreticulin to the cell surface which in turn functions as a signal for immunogenic cell death [251, 252]. Calreticulin surface expression was previously reported in PHA treated trout PBLs [117], therefore it can be speculated that ERp57 can reach the cell surface by interacting with calreticulin.

Many studies in mammals have focused on some additional surprising functions and/or locations of ERp57 which are still not fully understood. The presence of ERp57 in nuclei was first observed in rat spermatids by immunofluorescence and later evidence suggested that ERp57 interacts with the transcriptional factor STAT3 inside the nucleus [253, 254], possibly playing a role in STAT3 signaling [255]. In this study, ERp57 expression was shown to be induced for the first time by phytohemagglutinin and synthetic double stranded RNA, however not during single stranded RNA viral infection with VHSV IVa.

Furthermore, ERp57 was found to be secreted from mouse embryonic fibroblastic cell line 3T3 [256] and also from trout cells, as ERp57 protein was detected in the conditioned media of non stimulated RTS11 cells (data not shown). The possible role of such secretion is unclear and and additional studies using different stimuli should be undertaken to elucidate this phenomenon.

7.1.2 Possible interaction in the PLC

One of the most investigated functions of ERp57 is its role during the assembly of the peptide loading complex in the ER. It has been shown in mammals that tapasin alone cannot assemble with the MHC class I and facilitate peptide loading but only tapasin conjugated to ERp57 can do this [257]. The tapasin-ERp57 heterodimer formation is mediated by a disulfide bond formed between cysteine 57 in the thioredoxin domain of ERp57 and cysteine 95 in the luminal domain of tapasin, and this complex sequester almost all of the cellular pool of tapasin [40]. Interestingly, Cys57 of ERp57 is conserved in teleost species, however the conserved Cys95 of tapasin appears to be absent in fish and in birds which intuitively suggest on a lack of tapasin-ERp57 within the PLC and predict a much less stringent assembly for the MH class I that follow a normal ER quality control. Nevertheless, closer observation in the trout, salmon and the zebrafish sequences indicate a unique shared cysteine residue in position 57 downstream of Cys95 which may be potentially used to interact with ERp57. Attempts during this project to detect ERp57 and tapasin interaction by immunoprecipitation were unsuccessful and mostly inconclusive. However, when detergent extraction was performed in the presence of MMTS to protect native disulfide bonds [40, 37], the ERp57-tapasin interaction under non reducing conditions was detected as a 110 kDa conjugate, similar to mammals. These results suggest indirectly that trout ERp57 may be recruited to the peptide loading complex through its interaction with tapasin. Additional experiments are required to validate this interaction such as using purified recombinant proteins of trout ERp57 and tapasin to examine conju-

gate formation *in vitro* combined with site directed mutagenesis of the putative interacting cysteine residues. In addition, detecting the levels of conjugate formation during viral infection and comparing them to the normal levels could potentially provide new insights on the functional role of such interaction during viral infection.

7.2 Calnexin

7.2.1 Functional charectrization

Calnexin is a conserved lectin chaperone involved in glycoprotein folding in the ER. In this study, the first identification and characterization of calnexin in salmonid fish was described. Two calnexin cDNA clones were reported and southern bloting suggested that calnexin gene is present in two copies in the trout genome, in contrast to its soluble homologue calreticulin which appears as a single copy gene in trout [117]. Sequence analysis demonstrated that only one of the two cDNA clones encodes a conserved putative ER retention motif followed by a conserved serine phosphorylation site. Northern blotting suggested that there is one prominent expressed transcript for calnexin along with a smaller, lower expressed transcript, which appears exclusively in immune system organs. Exposure of RTS11 cells to calcium ionophore A23187 demonstrated the induction of the lower sized transcript, whereas the prominent transcript remained unchanged. These findings seem to be further supported at the protein level, which suggest that in trout there are two similar sized versions for calnexin protein in which only the smaller sized versions is induced under ER stress conditions. As both reported cDNA clones putatively encode for similar weight proteins, it could be speculated that post translational modification such as phosphorylation could account for the differences in the molecular weight possibly due to the different number of phosphorylation sites encoded by both calnexin genes. Future studies, should focus on cloning the promoters of both calnexin genes to identify ER stress elements (ERSE) responsible for such unique regulation during ER stress. However the possibility of post translational cleavage which results in two calnexin protein forms, cannot be ruled out. It has been shown that the C terminal domain of mouse calnexin can be cleaved by specific caspases under diverse ER stress and apoptotic conditions and can play a role in attenuation of apoptosis [132]. However, the conserved caspase cleavage site was not found in the trout sequences.

7.2.2 Interaction with the MH class I

Calnexin was first identified as a protein that associates with free MH class I in the ER [28]. The functional role of calnexin in MHC class I folding is controversial and functional discrepancies were found between the human and mouse proteins [23, 141, 30] despite their high percentage of sequence identity. Experiments with calnexin deficient cell lines demonstrated that calnexin is not absolutely required for the MHC class I folding and suggested that it might be replaceable by other chaperones, however other experiments in mice showed that calnexin is important for MHC class I folding. These discrepancies further support the importance in elucidating the function of calnexin in other vertebrae species to better understand how the MHC class I folding has evolved to facilitate optimal peptide loading. It has been shown that calnexin not only binds the free class I heavy chain but also is the only component from the peptide loading complex that associates with both the MHC class II α and β chain subunits [139]. In channel catfish, calnexin interaction with the MHC class II has been identified with the non glycosylated α chain and the glycosylated β chain [148], however the binding to the MHC class I heavy chain was not addressed in that study. Our preliminary data indicate that both protein versions of calnexin can bind the MH class I, during VHSV IVa infection, however, this association appears to be more prominent with the higher molecular weight version of calnexin. In addition, ERp57 interacts with the smaller version of calnexin protein under viral stimulating conditions. Future studies should examine if this interaction is also maintained during normal conditions. Generating specific antibodies against the native MHC class I could help to resolve if calnexin binds to the free heavy chain alone or the mature class I heterodimer. Interestingly, calnexin interaction with the G protein of vesicular stomatitis virus (VSV) which belongs to the rhabdoviridae family [143] has also been described. Attempts in this study to detect the interaction between the G protein of the fish rhabdovirus, VHSV, and immunoproteins were not successful, however further attempts should be pursued to reveal if this interaction can occur.

7.3 Tapasin

7.3.1 Tapasin inducible expression

Tapasin is a transmembrane glycoprotein that exclusively interacts with the MHC class I heterodimer. Unlike the other proteins in the peptide loading complex, which display high

sequence identity with their mammalian homologues, tapasin is less conserved and displays only 31% sequence identity with its human homologue. However, structural similarity, linkage to the MH class I region and induced transcript expression during viral infection suggests its function is conserved in trout [86, 122]. In this study, polyclonal antibodies against trout tapasin were generated in order investigate its regulation during viral infection and its putative interaction with the MH class I molecule. This study demonstrate that trout tapasin is a 48 kDa protein that is mainly expressed in immune system organs and is most abundant in the RTS11 cell line. Tapasin protein expression was enhanced in RTS11 cells upon exposure to synthetic double stranded RNA as well as during infection with chum salmon virus (CSV) and viral hemorrhagic septicemia virus (VHSV IVa), which correlates with enhanced transcription.

7.3.2 Tapasin protein interactions

This study shows for the first time in fish that tapasin interacts with the MH class I heavy chain and with the TAP under normal and viral infected conditions which suggests that rainbow trout tapasin might have a conserved role in antigen presentation as in mammals. However, the specificity of tapasin interaction with specific MH class I allelles needs to be explored using allele-specific MH class I monoclonal antibodies. Future studies should reveal its possible functions in stabilizing TAP and in selecting high affinity peptides. Two independent studies have recently demonstrated a powerful methodology to study the role of tapasin in selection of high affinity peptides. One approach used recombinant purified MHC class I and tapasin which were fused by leucine zipper at their c terminal to generate a strong interaction. Using a cell free approach, the binding kinetics of fluorescently labelled peptides was examined. The authors showed that tapasin initially bound a diverse pool of fluorescently labelled peptides, however only those with minimum binding energy threshold were retained [258]. In another cell free approach, the reconstitution of purified recombinant ERp57-tapasin into tapasin deficient cell lysates was employed and a complex with the MHC class I recombinant protein and radiolabelled peptides was applied, the binding was determined by using immunoprecipitation of the MHC class I and quantifying the amount of radiolabelled peptides. This study showed that only when tapasin is linked to ERp57, can it mediate the loading of high affinity peptides [257].

7.3.3 A possible truncated version of tapasin

Interestingly, screening of various rainbow trout cell lines and trout tissues for the expression of tapasin protein detected not only the predicted 48 kDa band but also an additional 20k Da band which remained relatively unchanged during poly I:C stimulation and viral infection. Since tapasin antibodies were generated against its cytoplasmic tail which binds TAP, these results imply a possible shorter version of tapasin which contains the cytoplasmic domain that could interact with TAP. Interestingly, in addition to the 110 kDa heterodimer of ERp57 and tapasin, an additional 75 kDa band was detected in RTS11 cell lysates pretreated with MMTS by both by tapasin and ERp57 antibody. This finding could imply that ERp57 interacts with a truncated tapasin version, which in turn might be recruited to the MHC class I peptide loading complex. Tapasin splice variants have been recently described in human melanoma cell line and subsequently in human cells infected with HCMV which results in deletion of exon 3 yet contains an intact cytoplasmic tail. Transfection of this splice variant into tapasin deficient cells could not restore MHC class I expression on the cell surface [194], yet demonstrated that both the alternative splice variant and the full length protein can bind in the PLC and therefore might influence its function [259].

Although, alternative splice variants for tapasin could not be found in this study, identification of the 20 kDa protein by immunoprecipitation followed by mass spec analysis was performed, and suggested on a possible tapasin peptide derived from the 20 kDa gel band. However, these results should be further confirmed by pull downs of tapasin using cell lines that only express the 20 kDa band such as RTtestis and RTovarian fluid. These trout cell lines could also be used to explore the possible role of the 20 kDa band in the peptide loading complex, in particular its binding to TAP and the MHC class I.

Interestingly, a few viral proteins were shown to interfere with tapasin function [59, 58], therefore it could be speculated that if the 20 kDa band is indeed a shorter version of tapasin, it could act as attenuate antigen presentation by interfering with the recruitment of the full length tapasin to the PLC.

7.4 β2m and MH class I expression during viral infection

7.4.1 β2m secretion

β2m is an 11 kDa protein that associates non-covalently with the MH class I heavy chain on the cell surface of all nucleated cells. β2m, unlike the heavy chain does not contain a transmembrane domain and can be released into the extracellular space during normal turnover of MH class I receptors on the cell surface [214, 215, 233]. High secretion levels have been observed in the urine and plasma of patients with numerous human diseases [260, 261, 262]. In this study, the secretion of β2m into the culture media of RTS11 cells was examined both under normal and VHSV IVa infected conditions. Under normal conditions β2m release was detected in the culture supernatants and this was significantly enhanced when RTS11 cultures were infected with the VHSV IVa, seven days post infection. The functional role of this secretion is still undetermined.

On one hand, the shedding of β2m can reflect the normal turnover of the MH class I receptor on the cell surface and can be directly correlated with expression levels and stability of the heterodimer on the cell surface. On the other hand, it could be speculated that β2m by itself has a unique regulatory role during immune response which is independent from its role in antigen presentation. Previous studies have demonstrated that β2m can stimulate chemotactic responses of hematopoietic cells such as T cell precursors *in vitro* [263, 236] indirectly suggesting a cell surface receptor for this molecule. In contrast, exposure of dendritic cells to a high concentration β2m into the culture media showed surprising negative regulatory effects on MHC class I levels and antigen presentation in these cells [238]. To elucidate the function role of such secretion in rainbow trout, future studies should examine *in vitro* the possible effects of trout recombinant β2m on the proliferation, differentiation and chemotaxis in rainbow trout cell lines combined with in vivo studies to determine the potential use of monitoring serum β2m levels as a biomarker for fish viral infection.

7.4.2 Expression of the MH class I during dsRNA stimulation

In this study, the regulation of the MH class I protein was examined in RTS11 cells upon exposure to synthetic dsRNA and upon infection with CSV, a dsRNA virus which belongs

to aquareovirus family [182]. Although this virus does not cause any pathogenesis in salmonid fish [183], it has been demonstrated *in vivo* that pre-exposure of rainbow trout to CSV offered protection from a subsequent infection IHNV which is a major viral threat to salmonid fish in North America [184]. The mechanisms of such protection were later revealed by an in vitro study in RTS11 cells which showed that both poly I:C and CSV can induce the expression of anti-viral genes such as MX and vig1 [185]. In this work, exposure of RTS11 cells to poly I:C, induced the expression of the glycosylated MH class I heavy as early as six hours post treatment, whereas the induction of the MH class I heavy chain in CSV infected cultures only occurred three days post infection and increased to the highest levels on day 14. It could be suggested that the mechanisms that are involved in viral entry, uncoating and transcription may partly delaying the interferon response compared with the synthetic double stranded RNA, which is taken up by scavenger receptors. These results suggest that both poly I:C and CSV might be used as adjuvants in vaccines for salmonids to stimulate antigen presentation and T cell mediated responses.

7.4.3 Temperature dependent regulation of MH class I expression during VHSV infection

Viral hemorrhagic septicemia (VHS) is an infectious fish disease which is responsible for the mortality of many marine and freshwater species [201]. The cause of the disease is VHSV which belongs to the rhabdoviridae family. In contrast to the chum salmon virus, VHSV is an enveloped virus and contains a single stranded, non-segmented RNA genome. By using the powerful suppressive subtractive hybridization (SSH) technique, four novel interferon responsive genes along with three chemoattractant molecules were identified following VHSV infection in rainbow trout leukocytes. Surprisingly, neither the MHC class I molecules nor the genes encoding the inducible proteasomal subunits were retrieved in the SSH [264]. However, evidence for the important functional role of the MHC during VHSV infection was clearly identified in a later study which showed isolated PBL from VHSV infected trout could kill MHC class I matched rainbow trout gonadal fibroblastic like cells (RTG-2) infected with VHSV. These finding highlights the significant role of cellular immunity in providing protection against VHSV infection.

In this study, MH class I regulation was examined upon infection with VHSV at two different temperatures: 14°C and 2°C to reveal potential mechanism responsible for fish susceptibility to rhabdoviral infection during spring and fall [265, 217]. On one hand, when

RTS11 were infected with VHSV at 14°C, class I heavy chain protein levels increased and enhanced cell surface expression was observed by immunofluorescence. On the other hand, when RTS11 infected cultures were maintained at 2°C no induction of the MH class I heavy chain was observed during infection and cellular expression levels remained similar to the control at at 14°C. Although β2m levels in both infected and non-infected 2°C cultures were also maintained, β2m secretion on the other hand was completely impaired both in control and infected cultures. These results suggest that MH class I surface expression might be compromised at low temperature which in turn could reduce antigen presentation and therefore allow the virus to replicate without being eliminated. This scenario could possibly contribute to the severity of VHS disease during changing water temperature. Future studies should further validate these results by flow cytometry and immunohistochemistry and look for the mechanisms that are responsible for such suppression in cold temperatures.

7.4.4 The peptide loading complex

The proposed model for the peptide loading in fish includes five proteins that were shown to comprise the PLC in humans: tapasin, TAP, MH class I, ERp57 and one of the two lectin chaperones (calnexin/calreticulin). However, an additional 20 kDa protein which appeared to interact with ERp57 is a possible unique component in the PLC of fish (Figure 7.4.1). Although, important interactions were identified in this study, the functional significance of such interactions on antigen presentation in fish remains unresolved. By using specific siRNAs that inhibit the expression of components such as tapasin, TAP and ERp57 or by using ER glucosidase inhibitor which prevent the binding of calnexin/calreticulin to their substrates, the impact of such interactions on MH class I surface expression and CD8+ response can be specifically explored.

Importantly, the binding of tapasin to the MH class I was explored in this study in trout immune system cells which express a unique set of MH class I alleles. It remains unclear if this binding occurs *in vivo* in fish that express a different repertoire of MH class I alleles. Future studies should focus on development of monoclonal antibodies against various MH class I proteins in order to determine which MHC class I proteins interact with tapasin and examine if their binding to tapasin can dictate an increase on the cell surface of a specific MH class I protein. Exploring these interactions can also assist in revealing which MH class I alleles might be important for antigen presentation in fish.

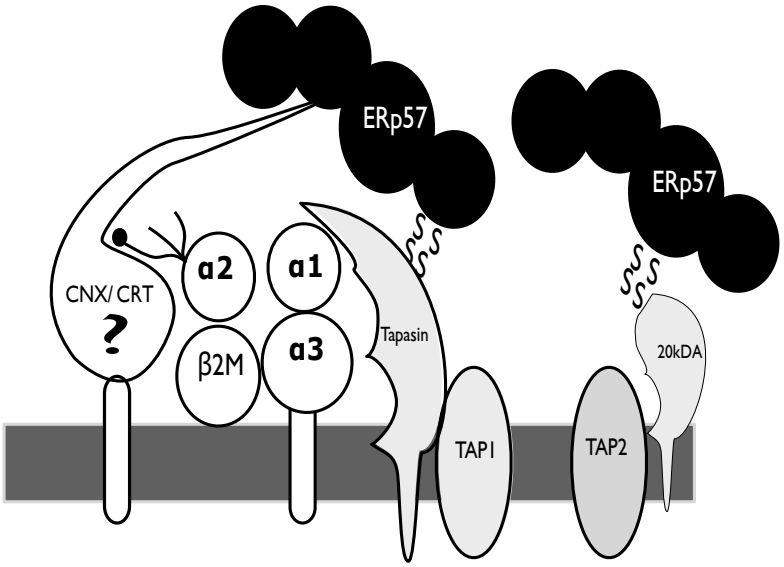

Figure 7.4.1: **Proposed model for the PLC in fish**
The possible components in the peptide loading complex in fish include tapasin conjugated to ERp57 via disulfide bond, TAP, MH class I and one of the lectin chaperones calnexin/calreticulin. A possible truncated version of tapasin which can interact with ERp57 and TAP is proposed to play a regulatory role in the formation of the PLC in fish.

7.5 Summary

This study demonstrates the identification and functional characterization of the peptide loading complex proteins and chaperones. In particular, calnexin and ERp57 in rainbow trout which share high sequence identity with their human homologues were characterized. Both the ERp57 and calnexin genes were found to be duplicated in trout and both appear to encode pairs of similar proteins with unique C terminal domains which suggest their unique functions. Furthermore, this study shows that trout tapasin protein is induced during CSV and VHSV IVa infection and interacts with the glycosylated MH class I receptor, TAP and ERp57, which provides the first direct evidence for its possible conserved role in antigen presentation in fish.

In addition, the lectin chaperones, calnexin and calreticulin were shown to interact with MH class I receptors in normal conditions which suggests their possible role in supporting the MH class I folding in fish as they do in mammals. Importantly, an additional 20 kDa protein was detected by the tapasin antibody which is speculated here to be a truncated tapasin version which interacts with ERp57 and could be involved in regulating antigen presentation in fish. Lastly, β2m secretion, a measure of MH class I turnover on the cell surface, was detected under normal conditions in RTS11 conditioned media and appeared

to enhance upon infection with VHSV IVa, along with higher cellular expression of MH class I. This secretion was severely reduced at 2°C, in both control and infected cultures, although cellular protein levels of β2m were maintained. These findings suggest that trafficking of the MH class I molecule may be abolished at low temperatures which can contribute to the susceptibility to pathogens in the cold.

This work opened new future avenues to explore mechanisms of peptide selection, viral evasion and importantly modes of regulating the MH class I surface expression in fish. Understanding such mechanisms can assist in understanding the fish immune response and provide important knowledge for aquaculture that can assist in vaccine design and disease prevention.

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