2021

**Functional Characterization of Infectious Hematopoietic Necrosis Virus Matrix Protein in Host Cellular Responses**

Jeffery Ringiesn  
*Wright State University*

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FUNCTIONAL CHARACTERIZATION OF INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS MATRIX PROTEIN IN HOST CELLULAR RESPONSES

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

by

JEFFERY RINGIESN
B.S., Shawnee State University, 2012

2021
Wright State University
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Jeffery Ringiesn ENTITLED Functional Characterization of Infectious Hematopoietic Necrosis Virus Matrix Protein in Host Cellular Responses BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Ringiesn, Jeffery. M.S, Department of Biological Sciences, Wright State University, 2021. Functional Characterization of Infectious Hematopoietic Necrosis Virus Matrix Protein in Host Cellular Responses

Infectious hematopoietic necrosis virus (IHNV) is a deadly fish pathogen that poses a global threat to aquatic ecosystems and the aquaculture industry. For decades, research has focused on developing vaccine therapeutics utilizing a variety of techniques and strategies. While these studies have met with some success in identifying potential vaccine targets that provided protective immunity, a commercially viable IHNV vaccine is currently unavailable. Here we explore the relationship between the structure and function of the IHNV matrix (M) protein through the introduction of mutations that reduce anti-host effects, with the goal of developing a novel recombinant IHNV with reduced pathogenicity that provides protective immunity. Our data suggest that the N- and C-termini of IHNV M contribute to its antitranscriptional effects and protein stability and are therefore functionally important. IHNV M mutants that exhibited reduced anti-host effects, but retained wild type protein expression levels, are prime candidates for additional study and incorporation into a novel recombinant IHNV system established in these studies. Overall, our studies highlight the feasibility of developing novel IHNV antiviral candidates through a mutational strategy that will yield additional information about viral protein function.
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<tr>
<td>a.a.</td>
<td>Amino Acid</td>
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<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
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<tr>
<td>CPE</td>
<td>Cytopathic Effects</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
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<tr>
<td>dsRNA</td>
<td>Double-stranded RNA</td>
</tr>
<tr>
<td>EPC</td>
<td><em>Epithelioma papulosum cyrinid</em></td>
</tr>
<tr>
<td>G</td>
<td>Glycoprotein</td>
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<tr>
<td>HCV</td>
<td>Hepatitis C Virus</td>
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<tr>
<td>HdvRz</td>
<td>Hepatitis Delta Virus Ribozyme</td>
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<td>Hammerhead Ribozyme</td>
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<td>Horseradish Peroxidase</td>
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<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IHNV</td>
<td>Infectious Hematopoietic Necrosis Virus</td>
</tr>
<tr>
<td>IKKe</td>
<td>Inducible IκB Kinase ε</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon Regulator Factor</td>
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<tr>
<td>ISG</td>
<td>IFN Stimulated Gene</td>
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<tr>
<td>ISGF3</td>
<td>IFN-stimulated gene factor 3</td>
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<td>JAK</td>
<td>Janus Kinase</td>
</tr>
<tr>
<td>L</td>
<td>Polymerase</td>
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<tr>
<td>M</td>
<td>Matrix Protein</td>
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<tr>
<td>MAVS</td>
<td>Mitochondrial Antiviral Signaling Protein</td>
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<tr>
<td>N</td>
<td>Nucleoprotein</td>
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<tr>
<td>NEMO</td>
<td>NF-κB essential modulator</td>
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<tr>
<td>NV</td>
<td>Non-virion Protein</td>
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<tr>
<td>P</td>
<td>Phosphoprotein</td>
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<td>PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
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PAMP .................................................. Pathogen Associated Molecular Pattern
PRR .................................................. Pathogen Recognition Receptor
PS .................................................. Penicillin-Streptomycin

RABV .................................................. Rabies virus
RdRP .................................................. RNA Dependent RNA Polymerase
RIG-I .................................................. Retinoic Acid Inducible Gene I
rIHNV .................................................. Recombinant IHNV rIHNV_pCI Plasmid
RLR .................................................. Retinoic Acid-Inducible Gene-I-Like Receptor
RLU .................................................. Relative Luciferase Units
RTgill-W1 ............................................ Rainbow Trout Gill

SEM .................................................. Standard Error of Mean
SHRV .................................................. Snakehead Rhabdovirus
ssRNA .................................................. Single Stranded Ribonucleic Acid

T7 RNAP .............................................. T7 RNA Polymerase
TBK1 .................................................. TANK Binding Kinase I
TLR .................................................. Toll-Like Receptor
TNF .................................................. Tumor Necrosis Factor
TRAF .................................................. TNF Receptor Associated Factor
TYK2 .................................................. Tyrosine Kinase 2

VHSV .................................................. Viral Hemorrhagic Septicemia Virus
VSV .................................................. Vesicular Stomatitis Virus
List of Symbols

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<thead>
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<tr>
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<td>μ</td>
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DEDICATION

This is dedicated to my loving partner, Catherine. Your support, love, and patience push me to be better day after day. And to our fur-babies: Piper, Mochi, and Hideo.
CHAPTER 1: INTRODUCTION

1.1 General Introduction

Sources of sustainable food products, particularly seafood, are more essential than ever as global population and demand increase every year. In order to maintain supply for this ever-increasing need, countries are driven to expand the number of fisheries and aquaculture facilities available. In 2018, the worldwide production of all commercial aquatic animals reached an all-time high of 178.5 million tonnes, a 3.4% increase from 2017, with an estimated value of $401 billion (1). However, as this industry continues to develop, so does the increase in international trading and shipping, promoting the spread of aquatic pathogens (2). The globalization of pathogenic bacteria and viruses present a danger not only to commercial aquaculture facilities, but risks expanding to wild populations as well. For at least seventy years the viral pathogen Infectious Hematopoietic Necrosis Virus (IHNV) has led to devasting losses within the aquaculture industry and significantly impacted aquatic ecology. With a mortality rate up to 90% or more, IHNV is responsible for catastrophic outbreaks within salmonid fishes around the world, affecting the global farmed salmonid market that is valued at more than $17.5 billion (3). Together, these factors demonstrate that IHNV is a key threat to aquatic ecology and to the rapidly expanding multibillion-dollar fishing industry. As such, a better understanding of IHNV biology and pathology is critical for developing novel therapeutics to facilitate reduction of this global threat.
1.2 Infectious Hematopoietic Necrosis Virus

1.2.1 Spread and Etiology

First recorded in the early 1950s among juvenile sockeye salmon in western North America, Infectious Hematopoietic Necrosis Virus (IHNV) infects numerous salmonid species, such as Atlantic salmon (Salmo salar) and rainbow trout (Oncorhynchus mykiss) (2, 4). Although IHNV was initially identified within the Pacific northwest, over time the virus spread to Europe and Asia via the commercial fish trade, infecting various domestic and wild salmonid species. It was not until 1971 that IHNV was first characterized in Asian countries, when diseased sockeye salmon (Oncorhynchus nerka) were imported from the United States to Japan (5). Sixteen years later in 1987, IHNV was detected in Europe for the first time, within France and Italy (6, 7). IHNV has subsequently spread throughout the Northern Hemisphere, infecting salmonid species within at least eighteen countries across North America, Asia, and Europe (8).

IHNV has been reported in at least 28 freshwater and marine species, with juvenile fry being the most highly susceptible population, as older fish are typically more resistant to disease (2). Infected fish exhibit symptoms such as lethargy, abnormal behavior, discoloration of skin and gills, exophthalmia, and hemorrhaging of mucus membranes (Fig. 1) (8, 9). Mortality depends on numerous factors such as host species, rearing conditions, environmental temperature, and viral strain, but mortalities as high as 100% in juveniles and 15-80% in adults have been reported (10). Mortality typically occurs within five days post initial infection in susceptible hosts, where the virus has spread from epithelial tissue
to vital organs such as kidney, spleen, thymus, liver, pancreas, heart, and brain. IHNV is readily transmitted horizontally between hosts via contact with mucosal secretions, urine, and sexual fluids with the primary portals of entry considered to be the gills and at the bases of fins. Vertical transmission via egg-associated infections has also been reported, originating from eggs that were reared in a virus-free environment (11). Once the virus has been shed, it can survive in marine or freshwater environments for at least a month in temperatures ranging from 8-18°C (8).
Figure 1. **Gross pathologies of IHNV infection in juvenile fry trout.** Arrows and circles indicate various signs of infection such as skin darkening (a and b), abdominal distension (a & c), exophthalmia (a & b), hemorrhage of the eye (a and b), pale gills (e), ulceration of the snout (c and d) and visceral pallor with yellowish fluid in the intestine (e) (Adapted from Ahmadivand et al., 2017) (9).
1.2.2 Nomenclature and Classification

IHNV is a member of the genus *Novirhabdovirus*, within the *Rhabdoviridae* family, which is characterized by the presence of a unique nonstructural nonvirion (NV) gene (12). IHNV isolates have been classified in various ways, but the method most widely used for strain differentiation is phylogenetic classification based on genomic sequence analysis. Within the Pacific northwest of the Unites States and Canada, phylogeography of IHNV supports classification of three distinct genogroups: Upper, Middle, and Lower (U, M, L) (Fig. 2) (13). Limited genetic diversity has been observed within U and L genogroups, indicative of evolutionary equilibrium. However, genogroup M exhibits higher nucleotide sequence diversity, indicating ongoing adaptation to novel conditions (13). Isolates from Asia and Europe are classified within their own separate genogroups, J and E, respectfully. While evidence suggests that these genogroups originated from the United States or Canada, their introduction into foreign ecosystems containing novel host species and environments has led to an increased selective pressure on IHNV, causing rapid evolutionary progression (5, 14).
Figure 2. Geographical localization of North American IHNV genogroups. Circles represent general areas where different variants have been characterized by genome sequencing. Genogroup U spans the greatest area, localizing in the northern, “upper”, regions of North America from the northern-Alaskan to mid-Oregon coast. The M, or “middle”, genogroup viruses are located more inland, collecting within southern Idaho, the Columbia River Basin, and the Washington Coast. The “lower”, or L, genogroup is located the furthest south, ranging from southern Oregon to central California (Adapted from Garver and Wade, 2017) (15).
1.2.3 IHNV Structure and Replication

Like all other Rhabdoviruses, IHNV is an enveloped, negative-sense, single-stranded RNA (-ssRNA) virus harboring a non-segmented RNA genome of approximately 11.1 kilobases (kb) (Fig. 3). The IHNV genome encodes for five structural proteins: nucleocapsid protein (N), polymerase-associated phosphoprotein (P), matrix protein (M), surface glycoprotein (G), and viral RNA polymerase (L) (16). IHNV also encodes one nonstructural protein, the nonvirion (NV) protein, which is unique to the Novirhabdovirus genus (12).

The IHNV viral life cycle is similar to other Rhabdoviridae members (Fig. 4). Briefly, the virus is packaged into a helical ribonucleocapsid core surrounded by a host-derived lipid bilayer envelope. This core consists of the -ssRNA viral genome, in addition to the N, P, and L proteins. Viral attachment occurs via interaction between host cell receptors and the G protein, promoting viral uptake through endocytosis (17). Within the cell, the ribonucleocapsid core is released into the cytoplasm following vesicular acidification, mediated by lysosome/endosome fusion (18). Once in the cytoplasm, the N protein dissociates from the viral genome, allowing mRNA transcription by a viral RNA-dependent RNA polymerase (RdRp), encoded by the viral L gene (19). A viral replication complex is formed with the addition of various host factors and viral P protein, stabilizing and activating the L protein by aiding in the binding of the replication complex to the leader segment of the -ssRNA genome, thus initiating transcription of a positive-sense mRNA transcript for each viral gene (20). Viral transcription occurs in a 3’ to 5’ direction, with
**Figure 3. Schematic of IHNV viral structure and genome.** IHNV is an enveloped, bullet shaped Novirhabdovirus that contains a linear, single stranded negative sense RNA genome that encodes for five structural proteins: the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and a RNA dependent RNA polymerase protein (L). IHNV’s genome also encodes one nonstructural protein, the nonvirion (NV) protein (Adapted from Pore, 2012) (21).
Figure 4. Replication cycle of IHNV. Viral glycoprotein interacts with host cell-surface receptors, initiating endocytosis and IHNV entry (1, 2). Following endocytosis, acidification of the vesicle occurs due to lysosome fusion (3), causing a release of the viral capsid into the cytoplasm through a conformational change in the viral G protein (4). mRNA transcription from the negative sense single stranded RNA genome begins (5), however viral polymerase slippage causes more copies of 5’ mRNA transcripts compared
to 3’ transcripts (6). Translation of viral mRNA occurs through host machinery. G protein is produced within the rough endoplasmic reticulum (7). Once enough N protein is produced, it binds to the viral genome, inducing a functional shift of the viral polymerase, switching from mRNA transcription to genome replication (8a, 9). Packaged G protein is trafficked to the cell surface as a transmembrane protein where it will interact with cytoplasmic M protein (8c). Viral assembly and release occurs following genome coiling and organization, allowing the interaction between viral M and N proteins to promote viral budding (10) (Adapted from Pore, 2012) (21).
initiation only occurring at the 3’ end of the -ssRNA genome (22). During viral transcription, the genes located most 3’ are expressed at a higher copy number compared to the 5’ end. This phenomenon occurs due to polymerase slippage following polyadenylation signals and inter-segment regions, preventing the polymerase from progressing to the subsequent genes (23). The L protein will eventually switch from mRNA synthesis to positive-sense viral genome replication once an abundance of N protein is produced to coat the original -ssRNA viral genome. New copies of the -ssRNA viral genome are then transcribed from the recently replicated positive-sense RNA template, and formation of the viral ribonucleocapsid begins as accumulated N protein binds to the newly synthesized -ssRNA genome (22, 24). Following translation, transmembranal G protein is packaged within vesicles bound for cell surface expression following processing within the endoplasmic reticulum and Golgi apparatus. Once integrated to the host cell membrane, the cytoplasmic exposed portion of the G protein interacts with M protein, providing scaffolding for eventual viral budding (25). The ribonucleocapsid, consisting predominately of N protein, binds with the membrane-associated M protein, creating a zipper-like effect that forms the mature bullet-shaped virion (26).
1.3 Host Innate Immune System

1.3.1 Viral Detection and the Type I Interferon Response

Eukaryotes have established complex immune responses to combat viral pathogens. Detection of viruses via the innate immune system begins with interactions between viral pathogen associated molecular patterns (PAMPs) and host pattern recognition receptors (PRRs) (Fig. 5) (27). PAMPs are highly conserved throughout nature, found on numerous organisms in addition to viruses, such as bacteria, fungi, and protozoa. During virus infections, PRRs known as toll-like receptors (TLRs) detect viral PAMPs on the host-cell surface or within endosomes (28). Two TLRs involved with the detection of viral pathogens, more specifically double and single stranded viral RNA, are TLR3 and TLR7, respectfully (29). Activation of these TLRs result in the induction of signaling proteins including tumor necrosis factor (TNF) receptor associated factors (TRAFs) family members such as TRAF3 (30). This induction ultimately leads to phosphorylation of transcription factors such as interferon regulator factors (IRF) 3 and 7 by the activated TRAF family member-associated NF-κB activator binding kinase 1 (TBK1) and inducible IκB kinase ε (IKKε). IRF3/7 phosphorylation promotes their translocation into the nucleus, initiating transcription of genes encoding interferons (IFNs) and various other pro-inflammatory cytokines (30).
Figure 5. Viral Detection and the Type I Interferon Response Pathway. Viral recognition initiating the Type I IFN response is dependent upon the detection and interaction between PAMPs, such as double and single stranded RNA (ds/ssRNA), and PRRs. Early in viral infections, TLRs recognize viral PAMPs within endosomes, while RIG-I-like receptors (RLRs) detect PAMPs with the cytoplasm. Following activation, TLRs activate TRAF family members, leading to phosphorylation and nuclear translocation of transcription factors IRF3 and IRF7. RLRs detect and interact with viral RNA, forcing a conformational change and promoting interaction with MAVS. Following activation, MAVS interacts with other members of the TRAF family, such as TRAF6. This
interaction promotes activation and translocation of downstream transcription factor NF-κB. Translocation of these various transcription factors induce upregulation of Type I IFN and other pro-inflammatory genes. Secreted Type I IFN acts upon neighboring cells through the binding of IFN receptors, IFNAR1 and IFNAR2. This interaction ultimately leads to the upregulation of various ISGs such as PKR, MX1, and ISG56 which all collaborate to achieve an antiviral state (Adapted from Nelemans T. and M. Kikkert, 2019) (29).
In addition to recognition by TLRs, viral single or double stranded RNA is detected within the host cytoplasm by retinoic acid inducible gene I (RIG-I)-like receptors (RLRs) (31). Following PAMP detection, RLRs undergo a conformational change that allows binding to a downstream adaptor protein known as mitochondrial antiviral signaling (MAVS) (32). Activated MAVS associates with various TRAF family members including TRAF2, TRAF3, and TRAF6 (33). Once associated with MAVS, TRAF6/2 activates the IKK protein kinase complex (34). This complex is comprised of IKKα, IKKβ, and NF-κB essential modulator (NEMO). After phosphorylation and degradation of IκB via activated IKK, NF-κB becomes activated and translocates into the nucleus, where it begins the transcription induction for various pro-inflammatory cytokines (30). This transcription activation via IRF3/7 and NF-κB is considered the “first phase” of the viral innate immune response within infected cells.

Class II cytokines known as IFNs are critical components of the cellular immune response, and are classified into three different types: I, II, and III. Type I IFNs are the most relevant and best characterized antiviral cytokines in mammalian cells, although type III IFNs also play a role in antiviral activity (35). This trait is also shared within fish cells, where type I IFNs exhibit coding sequence and crystal structure similarities with mammalian cells, although fish type I IFNs have distinct intron/exon structures as compared to mammals (36, 37). After the “first phase” of viral detection and response, IFNs are produced and secreted from the cells, triggering the “second phase” of the innate immune response (Fig. 5). Localized and circulating IFNs bind to the IFNAR receptor
complex consisting of IFNAR1 and IFNAR2 to initiate distinct signaling cascades (38). Conformational changes within the cytoplasmic domain of the engaged IFNAR complex activates associated Janus kinases (JAKs) tyrosine kinase 2 (TYK2) and JAK1, eventually leading to the phosphorylation of STAT1 and STAT2 (39). The formation of the multimeric transcription factor IFN-stimulated gene factor 3 (ISGF3) occurs through the association of STAT1, STAT2, and IRF9 (40). This ISGF3 transcription factor complex translocates into the nucleus and promotes the upregulation of dozens, if not hundreds of interferon stimulated genes (ISGs), promoting an induced antiviral state (41).

1.3.2 Viral Evasion and Manipulation of Host Immune Response

Overcoming the host innate immune response is critical for viruses to survive and propagate. Viruses have evolved numerous methods of evasion, some as simple as replicating rapidly enough to outpace host responses, or as clever as masking their PAMPs to avoid detection by the host (29). Viruses also have established mechanisms to directly inhibit components of the viral detection/response pathway to prevent type I IFN production. A common tactic utilized by many viruses is to inhibit the activation of IRF3 in some capacity, blocking the interaction between RIG-I and MAVS. This inhibition occurs often in Influenza A and Ebola virus infections where nonstructural viral proteins, NS1 and VP35 respectfully, directly interact with RIG-I and MAVS to prevent association (42, 43). An alternative approach is used by hepatitis C virus (HCV), where a viral protein cleaves MAVS off the mitochondrial membrane, preventing RIG-I binding (44).
Rhabdoviruses exhibit similar mechanisms of host immune response evasion. The P protein of Rabies Virus (RABV) inhibits IFN production and ISG activation by preventing IRF3 phosphorylation, thereby blocking dimerization and translocation into the nucleus (45). RABV P also prevents translocation of the ISGF3 transcription factor complex by inhibiting IFNAR-initiated JAK1/TYK2-mediated signaling (46). Other rhabdoviruses, including vesicular stomatitis virus (VSV) and viral hemorrhagic septicemia virus (VHSV), utilizes the M protein to suppress host responses more generally. In both cases, M shuts down host gene expression (47, 48). In VSV, this occurs through inactivation of transcription factor TFIID, preventing RNA Polymerase II mediated transcription. In addition to transcriptional inhibition, the VSV M protein also inhibits nuclear export of mRNA by interacting with nucleoprotein nup98 and export factor Rae1 (49, 50). Although this inhibition induces apoptosis, it still benefits the virus by preventing IFN or ISG signaling and reducing viral transcript competition for ribosomes. The nonstructural NV protein of VHSV facilitates numerous anti-host activities such as suppression of TNF-α-mediated immune responses (51), inhibition of host apoptosis (52), and antagonization of RIG-I-mediated IFN induction (53). More recently, VHSV NV protein was proposed to play a direct role in the PERK-eIF2α pathway, shutting off host translation and thus decreasing host IFN induction through increased eIF2α phosphorylation (54). Nevertheless, much remains unknown about Novirhabdovirus M and NV protein and their roles in host immune responses.
1.4 Genetic Manipulation of Nonretroviral RNA viruses

1.4.1 Recombinant Viruses and Reverse Genetic Systems

The ability to manipulate nucleic acids through recombinant DNA technology is an essential tool for elucidating the function of a particular gene product. Due to their small genome size, viruses are particularly susceptible to such modifications. Nonretroviral RNA viruses, however, face impediments to manipulation as they lack a DNA intermediate step within their replication cycle. Through the use of reverse genetics, researchers have achieved substantial advancements in the study of nonretroviral RNA viruses, which remains a critical tool for uncovering the functions of many viral genes by generating infectious clones of nonretroviral RNA viruses derived from a full-length cDNA template.

The first reported example of a recombinant single stranded RNA rhabdovirus from a full-length cDNA clone was achieved by Schnell et al., who developed a recombinant RABV (56). In that study, the authors expressed a full-length antigenomic RNA transcript derived from the RABV genome under the control of a T7 RNA polymerase (T7 RNAP) promoter, along with separate, plasmid-driven expression of viral N, P, and L proteins. Following this discovery, another single stranded RNA rhabdovirus, vesicular stomatitis virus (VSV), was also recovered following use of techniques (57, 58). Subsequent advancements and optimizations were made to improve recombinant virus recovery. For instance, researchers developed a host cell line that constitutively expressed T7 RNAP to aid in the recovery of measles virus, removing the requirement for a helper-T7 virus or
plasmid (59). This method was eventually adapted for use in VSV recovery by Harty et al. (60). Once free of T7 RNAP, researchers prioritized improving the recovery of single stranded RNA viruses through the use of autocatalytic ribozymes. Efficient RNA cleavage, leading to the production of exact end termini, an increased RABV recovery, significantly demonstrating that extra nucleotides present at the distal end of the viral minigenome affected viral rescue (61). Further optimization utilizing autocatalytic ribozymes occurred through the introduction of hammerhead ribozyme (HHRz) and hepatitis delta virus ribozyme (HdvRz), in conjunction with a RNA polymerase II system, for the recovery of RABV (62).

The development of a reverse genetics system for the recovery of Novirhabdovirus has also evolved over time, introducing the optimizations utilized in other single stranded RNA rescues. The first reverse genetics system for Novirhabdovirus was developed for snakehead rhabdovirus (SHRV) and employed a T7 RNAP expressing vaccinia virus (63). Utilizing the same method, a system was also developed for the recovery of another Novirhabdovirus, IHNV (64). Eventually this system was adapted to remove the requirement for the T7 RNAP and vaccinia virus by placing it under the control of a cytomegalovirus (CMV) immediate-early promoter for IHNV recovery (65). Soon after, this method of recombinant Novirhabdovirus recovery was used to create the first reverse genetics system for the rescue of VHSV and is now standard for the development of Novirhabdovirus reverse genetic systems (66).
1.5 Objectives and Specific Aims

The development of a novel vaccine that provides protective immunization against IHNV is critical for addressing the devastating global effects of this pathogen. While numerous attempts to develop a vaccine against IHNV have been made, no commercially available vaccine is currently available. Here, we aim to investigate the structure-function relationships of various regions within the IHNV M protein as a means of identifying regions that contribute to M’s pro-viral (structural) and anti-host (antitranscriptional) functions in order to eventually develop an attenuated recombinant virus using a novel reverse genetics system. Our central hypothesis is that an IHNV that is incapable of blocking host responses, perhaps coupled with a reduced replication capacity, will activate an immune response that leads to protective immunity. The increased understanding of IHNV M anti-host properties, and development of a novel reverse genetics system that promotes streamlined introduction of characterized M mutants, will lead not only to advancement in the knowledge of viral immune evasion, but also establish a new therapeutic target to aid in the reduction of this global threat.

In order to test the central hypothesis and achieve our overall objective, focus was placed upon the fulfilment of two specific aims. The first aim was to characterize functional sites within the IHNV M protein that impact host viral responses. We hypothesize that a mutant IHNV M with attenuated host suppression (antitranscriptional) ability could be identified in cell-based transfection studies using IHNV M in isolation. Previous data has
shown successful attenuation of VHSV M’s antitranscriptional effects through the introduction of specific a.a. changes (48). Those results suggest that introduction of similar mutations within IHNV M could lead to similar reduction of anti-transcriptional effects. The second aim was to engineer a novel IHNV reverse genetics system that permits simple and efficient introduction of characterized IHNV M (and NV) mutants to allow for efficient testing of both reduced anti-host effects and for the ability of the mutant M protein to support viral replication. While previous IHNV reverse genetic systems are well characterized, none of them currently allows for the rapid introduction of single, or multiple, viral gene mutations without reengineering the entire system.
Chapter 2: MATERIALS AND METHODS

2.1 Cell lines and culture maintenance

Epithelial cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA), including Fathead minnow (*Pimephales promelas*) *Epithelioma papulosum cyprini* (EPC) (ATCC: CRL-2872), and Rainbow trout (*Oncorhynchus mykiss*) gill (RTgill-W1) (ATCC: CRL-2523). Cell cultures were maintained in 25 cm$^2$ tissue culture flasks (CytoOne, Ocala, FL, USA) at 20°C, with L-15 Leibovitz media (HyClone, Marlborough, MA, USA) supplemented respectively with 1% Penicillin-Streptomycin (PS) solution (Corning, Corning, NY, USA), and with 2% (L15-2PS) or 10% (L15–10PS) fetal bovine serum (Corning, Corning, NY, USA) without CO$_2$. Complete media were sterile-filtered through a 0.2 μm cellulose nitrate membrane (Nalgene, Rochester, NY, USA).

2.2 IHNV plasmids and luciferase reporters

The IHNV M gene from M genogroup was cloned into expression plasmids for transient co-transfections experiments as previously described (67). Coding sequences were PCR-amplified using cDNA from archive viral stocks with specific primers (Table 1) to generate mutations within the IHNV M gene. PCR fragments were cloned into EcoRI and KpnI sites of pcDNA3.1(−)Myc/His A plasmid (Invitrogen, Waltham, MA, USA). Plasmids were amplified in *E. coli* DH5α cells and plasmid DNA was purified using Qiagen Plasmid Plus Midi Kit (Qiagen, Hilden, Germany), following the manufacturer’s instructions. All expression plasmids were sequence confirmed before use in transfection
experiments. The construction of other expression plasmids was previously reported (48). A luciferase reporter construct harboring the CMV enhancer/chicken β-Actin promoter, pCAG/luc plasmid (68), was purchased from Addgene (Plasmid #55764).

2.3 Transient Cell Transfection

Transfections were performed in L15–10, without addition of antibiotics, using the suitable transfection reagent for each cell line at a final 3:1 reagent volume (µl) to total DNA amount (µg). EPC cells were transfected using FuGENE HD Transfection Reagent (Promega, Madison, WI, USA), while RTgill-W1 were transfected using Via-Fect Transfection Reagent (Promega, Madison, WI, USA). Plasmid concentrations in all transfection experiments were equalized between samples by the inclusion of empty vector pcDNA3.1, which was also used in negative control groups. DNA mixtures were complexed with the respective transfection reagents in 37°C pre-warmed Opti-MEM I reduced serum medium (Gibco, Waltham, MA, USA), then incubated for 15 min at room temperature. 100 µl transfection doses were added to each confluent cell monolayer with 500 µl L15–10 in each well. 12-well plates (CytoOne, Ocala, FL, USA) were incubated at 20°C, without any further medium replacement or manipulation until the indicated sampling points.

2.4 Luciferase reporter assay

Luciferase assays were performed as described by Gorgoglione, et al. (67). Briefly, after the designated time post-transfection, cell monolayers in each well were gently washed with 1X PBS at 20 °C, then lysed for 15 min at RT in 200 µl of luciferase cell
Table 1. Primers for Cloning

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’ → 3’)</th>
<th>Restriction Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>IHNV M se</td>
<td>ACGAATTCATGTCTATTTTCAAGAGAGC</td>
<td>EcoRI</td>
</tr>
<tr>
<td>IHNV M as</td>
<td>CTTGGTACCTTTTCCCTCCCCCGCTTTTCGG</td>
<td>KpnI</td>
</tr>
<tr>
<td>IHNV M D63A se</td>
<td>GTCTCCGCTGAGGAGGTGAGGAGG</td>
<td></td>
</tr>
<tr>
<td>IHNV M E178A as</td>
<td>CCTACGATGGCAGCGCCAGATTTC</td>
<td></td>
</tr>
<tr>
<td>IHNV M E189A as</td>
<td>ACGGTACCTTTTCTTTCTCCCCGCTTTGCGGAGCGCAGAAGG</td>
<td></td>
</tr>
<tr>
<td>IHNV M Δ5N se</td>
<td>ACGAATTCATGAGAGGAGAAGAAGAAGAAGGAGGTC</td>
<td>EcoRI</td>
</tr>
<tr>
<td>IHNV M Δ10N se</td>
<td>ACGAATTCATGGTCTGATCCCTCCTCCTCCTCCT</td>
<td>EcoRI</td>
</tr>
<tr>
<td>IHNV M Δ20N se</td>
<td>ACGAATTCATGGGAGACGAGGAGGAGGAGG</td>
<td>EcoRI</td>
</tr>
<tr>
<td>IHNV M Δ5C as</td>
<td>ATCGGTACCCTTTGCGGAGCGCGCAGA</td>
<td>KpnI</td>
</tr>
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<td>ATCGGTACCAGGACAGGGCCAG</td>
<td>KpnI</td>
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<tr>
<td>IHNV M Δ20C as</td>
<td>ATCGGTACCATAAGTGTCTTTGACCCTC</td>
<td>KpnI</td>
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culture lysis reagent (Promega, Madison, WI, USA), used at 1X in molecular grade water (HyClone, Marlborough, MA, USA). Sampled 12-well plates were used immediately or kept at −20°C until assays were performed. From each cell lysate sample, 75 μl was collected for Luciferase reporter assay in 96-well white solid flat bottom opaque microplates (Greiner Bio-One, Kremsmünster, Austria), and 5 μl for Bradford assay in 96-well clear flat bottom microplates (Greiner Bio-One, Kremsmünster, Austria). The luciferase reporter assay was performed by adding to each sample 100 μl of a mixture containing: 51 μl of Luciferase assay ATP assay buffer [3.83mM EGTA (MP Biomedicals), 14.4mM Magnesium sulphate (Thermo Fisher Scientific, Waltham, MA, USA), 23.9mM Glycylglycine (Thermo Fisher Scientific, Waltham, MA, USA), 14.4mM Potassium phosphate dibasic (Thermo Fisher Scientific, Waltham, MA, USA), 0.98mM DTT (Dithiothreitol, Thermo Fisher Scientific, Waltham, MA, USA), 1.97mM ATP (Thermo Fisher Scientific, Waltham, MA, USA 0.33mM Coenzyme-A (Thermo Fisher Scientific, Waltham, MA, USA), in Milli-Q water], and 49 μl of Luciferin solution [1mM DTT, 25.1mM Glycylglycine, 0.27mM D-Luciferin (Thermo Fisher Scientific, Waltham, MA, USA), in autoclaved Milli-Q water]. Luminescence light emission was measured with a Synergy H1 microplate reader (BioTek Instruments, Winooski, VT, USA), setting top optics reading and luminescence spectral scanning gain/sensitivity to 135. The total protein load was measured by adding 5 μl of each cell lysate to 95 μl of Pierce™ Coomassie Plus (Bradford) solution (Thermo Fisher Scientific, Waltham, MA, USA) (diluted 50% in autoclaved Milli-Q water). Light absorbance values were immediately read at 595 nm with
a Synergy H1 microplate reader. Luminescence data, expressed as Relative Light Units (RLU), were normalized to lysate protein concentrations. The Relative Luciferase Activity (RLA) was calculated as the % ratio between stimulated (co-transfected with testing plasmids) and unstimulated (pcDNA3.1 alone) samples. All data shown are representatives of at least three independent experiments and presented as group means (±SEM).

2.5 Real-time quantitative PCR

RNA was isolated using Qiagen RNeasy Plus Mini Kit (Qiagen, Hilden, Germany), according to manufacturer’s protocol with supplemented Qiagen RNase-Free DNase (Qiagen, Hilden, Germany) treatment. For reverse transcription reactions, Optizyme M-MLV Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) was used to reverse transcribe total RNA isolated. Reverse transcription reactions were carried out by incubating RNA with 100 ng of random hexamer primer and water at 70°C for 10 min then briefly cooled on ice before addition of 5x MMLV RT buffer, MMLV RT, RiboLock RNase Inhibitor (Thermo Fisher Scientific, Waltham, MA, USA) inhibitor and 5 mM dNTPs (Thermo Fisher Scientific, Waltham, MA, USA). Samples were incubated at 42°C for 1 h. Undiluted cDNA samples were quantified using quantitative reverse transcription PCR (qRT-PCR) using 10 μl of 2x PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), 1 μl of undiluted cDNA, 50 ng of each primer pair (Table 2), and water to a total volume of 20 μl. Reactions and data collection were performed with a QuantStudio 7 Flex Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) using PowerUp SYBR Green manufacturer’s protocol for fast cycling mode with a
Table 2. Primers for RT-qPCR Analysis

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish β-actin se</td>
<td>AGACATCAGGGTGCATGGTTGGT</td>
</tr>
<tr>
<td>Fish β-actin as</td>
<td>GGGGTGCTCCTCTCTGGGGCAA</td>
</tr>
<tr>
<td>IHNV M RT se</td>
<td>ATCAAGGTGGCTGGAAGGAG</td>
</tr>
<tr>
<td>IHNV M RT as</td>
<td>GTAGGTCTGCATTGGGATCTTT</td>
</tr>
</tbody>
</table>
1 s denature time for a total of 40 cycles. Cycle threshold (Ct) values were obtained by an automated single point threshold within the log-linear range and normalized to β-Actin primer pair control signals. Expression levels were calculated using the 2-ΔΔCT method.

2.6 Immunoblotting

The expression of viral plasmids transfected in fish cells was assessed by Western blotting. Cell lysates were prepared and separated by SDS-polyacrylamide gel electrophoresis (PAGE), as previously described (54). Samples were electrophoretically transferred to Immobilon®-P PVDF membrane (MilliporeSigma, Burlington, MA, USA) and membranes were blocked with 5% (w/v) BSA/TBST (P-753, Boston Bioproducts, Ashland, MA, USA) for 1 h at room temperature. Primary antibodies, anti-Myc monoclonal (Myc, Invitrogen, Waltham, MA, USA), anti-β-Actin (Thermo Fisher Scientific, Waltham, MA, USA), or anti-Ubiquitin polyclonal (Invitrogen, Waltham, MA, USA) were diluted in TBST at 1:5000 and incubated overnight at 4°C. Membranes were incubated with the secondary antibody for 1 h at RT, using horseradish peroxidase (HRP)-conjugated Goat anti-mouse IgG1 (Invitrogen, Waltham, MA, USA) or anti-rabbit IgG, HRP-linked antibody #7074 (Cell Signaling Technology, Danvers, MA, USA) at 1:10,000 or 1:5000 dilution in TBST, respectively. Immunoreactive bands were visualized with SuperSignal™ West Pico PLUS chemiluminescent substrate (Thermo Fisher Scientific, Waltham, MA, USA) using Amersham Imager 600 (General Electric, Boston, MA, USA). Following exposure with anti-Myc or anti-Ubiquitin antibodies, membranes were stripped using Restore™ PLUS western blot stripping buffer (Thermo Fisher Scientific, Waltham,
MA, USA) for 10 min at room temperature, thereafter, blocked with 5% (w/v) BSA/TBST for 30 min at room temperature, and re-probed with the anti β-Actin antibody.

2.7 Protein sequence alignment

A.a. sequence alignment of Rhabdoviridae matrix protein was performed for the following species: Infectious hematopoietic necrosis virus (IHNV) (Sequence ID: ADB93798.1), viral hemorrhagic septicemia virus (VHSV) (Sequence ID: ADB93791.1), snakehead rhabdovirus (SHRV) (Sequence ID: NP_050582.1), vesicular stomatitis Indiana virus (VSV) (Sequence ID: NP_041714.1), and spring viraemia of carp (SVCV) (Sequence ID: NP_116746.1). Briefly, a.a. sequences acquired from NCBI Protein BLAST were aligned using a combination of software including ESPript (69), Clustal Omega (70), and PROMALS3D (71). General alignment, a.a. conservation, and figure format were derived using ESPript and Clustal Omega, while characterization of predicted secondary structures and a.a. properties were obtained through use of PROMALS3D.

2.8 Recombinant virus construction and recovery

Construction of a full-length cDNA clone of the IHNV 220-90 RNA genome (GenBank: GQ413939.1) was performed as previously described (65, 72). Briefly, the viral cDNA genome was synthesized and assembled by GENEWIZ (South Plainfield, NJ, USA) within the pCI vector (Promega, Madison, WI, USA), between the NheI and NotI restriction sites and under the control of a cytomegalovirus (CMV) promoter. The hammerhead ribozyme (HHRz) and hepatitis delta virus ribozyme (HdvR) DNA sequences were synthesized at the 5’ and 3’ ends, respectfully, of the viral cDNA genome. KpnI and
MluI restriction sites were engineered on either end of the M open reading frame (ORF), while SpeI and SacII restriction sites were introduced on either ends of the NV ORF. In addition to the construction and assembly of the recombinant IHNV rIHNV_pCI plasmid (rIHNV), supporting plasmids were also produced (GENEWIZ, South Plainfield, NJ, USA) containing the ORFs of N, P, and L viral genes within the previously described NheI and NotI restriction sites.

Generation of recombinant viruses through transient transfection was performed as previously described (65, 72). Briefly, EPC cells were transfected with the rIHNV plasmid (1 µg) construct along with combinations of N (0.5 µg), P (0.2 µg), and L (0.2 µg) pCI support plasmids. pcDNA3.1 plasmid encoding MYC and 6xHis-tagged Wild-type NV (NV-T) or untagged wild-type IHNV NV was also co-transfected (0.15 µg) within these combinations. Following transfection, cells were maintained in L15–10 at 15°C until virus-induced cytopathic effects (CPE) was observed, typically within 5 days post transfection.
Chapter 3: CHARACTERIZATION OF FUNCTIONAL SITES WITHIN IHNV MATRIX PROTEIN THAT IMPACT HOST VIRAL RESPONSES

3.1 Rationale

Our lab investigated the comparative effects of IHNV and VHSV genes on constitutive transcription and innate antiviral transcriptional responses in cold water fish cells (67). Those studies provided evidence that genes from these closely related Novirhabdoviruses shared similar host transcriptional modulatory effects, particularly by the M and NV genes where suppression and augmentation of constitutive host transcription occurred, respectively (Fig. 6). Our lab additionally investigated the effects of the VHSV M protein on host transcription inhibition by providing evidence that M suppresses RNA polymerase II (RNAP II) recruitment to a RNAP II-dependent promoter, and decreased RNAP II C-terminal domain Ser2 phosphorylation in a dose dependent manner (48). An attenuated VHSV containing a.a. point mutations found in an M variant from a less virulent strain of VHSV exhibited comparatively less potent antitranscriptional effects. Specifically, introduction of a.a. point mutations at residues 62 (D62A) and 181 (E181A) of the more potent VHSV M protein reduced its antitranscriptional effects as compared to wild type M (Fig. 7), but, interestingly, did not significantly hinder viral yield (48). These data suggested that anti-host function was separable from viral replication. With this information in mind, we tested whether similar mutations (Fig. 8) within the IHNV M protein also affected its function. While the primary and secondary protein structures of IHNV and VHSV are strongly conserved, the D62 and E181 residues are not found at these
exact sites within IHNV. However, similar residues exist in homologous regions within IHNV M, including D63. Two glutamic acid residues, E178 and E189 in IHNV M were found close to VHSV E181 and selected for mutation to alanine. A fourth residue, S184, was changed to a threonine through random mutagenesis via low-fidelity DNA polymerase.
Figure 6. Comparative modulation of host constitutive transcription by single VHSV and IHNV genes. EPC cells were co-transfected with pCAG/luc plus two doses of each (A) VHSV or (B) IHNV gene plasmid. Empty pcDNA3.1 plasmid vector was used for transfection balancing and baseline control. Luciferase activity was analyzed at 48 h post
transfection and RLU values were normalized to total protein concentration in each sample. Data are representative of three independent experiments. Values are group means ±SEM. *p < 0.05; **p < 0.01; ***p < 0.001 indicate significant differences from pcDNA control values as determined by one-way ANOVA and Fisher’s LSD test (Taken from Gorgoglione, et al. 2020) (67).
Figure 7. Impact of amino acid changes in VHSV M on transcriptional inhibition.

EPC cells were co-transfected with SV40/luc along with WT VHSV M, D62A mutant, or D62A E181A mutant for 24 h, followed by a luciferase reporter assay. Luciferase values were normalized to SV40/luc transfected sample and total plasmid amount normalized with empty pcDNA3.1 plasmid (Adapted from Ke et al., 2017) (48).
Figure 8. Schematic of IHNV M point mutations. IHNV M residues that were mutated are indicated by single letter a.a. abbreviations. Wild type M contains aspartic acid (D) at residue 63, glutamic acid (E) at residue 178, serine (S) at residue 184, and glutamic acid (E) at residue 189. Alanine (A) or threonine (T) substitutions are shown on the relevant mutant proteins.
3.2 Results

3.2.1 Impact of amino acid changes in IHNV M on transcriptional inhibitory function.

Suppression of host transcription by wild type IHNV M or M point mutants was assessed by using the constitutively expressed pCAG/luc reporter. EPC and RT-Gill cells were co-transfected with pCAG/luc and differing doses (0.05 µg and 0.1 µg) of each M mutant (D63A/E178A, D63A/E189A, E178A/E189A, and E63A/E178A/S184T/E189A) for 48 h, after which luciferase activity was quantified (Fig. 9). In EPC cells, all mutants exhibited dose-dependent antitranscriptional activity comparable to WT M, averaging roughly 80% transcription inhibition at 0.05 µg of plasmid and around 90% at 0.1 µg of plasmid. Similar effects were observed in RT-Gill cells, albeit with lower levels of antitranscriptional activity, around a 30% and 50% reduction respectfully (Fig. 9). These data suggest that the a.a. residues D63, E178, S184, and E189 are not critical for IHNV M antitranscriptional activity.
Figure 9. Impact of amino acid mutations in IHNV M transcriptional inhibition. (A) EPC and (B) RT-gill cells were co-transfected with 0.5 µg or 0.8 µg pCAG/luc, respectively, plus 0.05 or 0.1 µg of each IHNV M point mutation plasmid. pcDNA3.1
plasmid vector (pcDNA) was used for transfection balancing and baseline control. Luciferase activity was analyzed at 48 h post transfection and RLU s were normalized to total protein concentration in each sample, followed by normalization to pcDNA3.1 plasmid control. Data are representative of three independent experiments performed in triplicate. Values are group means ±SEM.
3.2.2 Comparative Rhabdovirus matrix protein alignment shows strong primary and secondary structure conservation.

M protein a.a. sequence alignments were performed on five different rhabdoviruses: IHNV, VHSV, SHRV, VSV, and SVCV using a combination of software including ESPript, Clustal Omega, and PROMALS3D (Fig. 10). The protein alignment identified regions of a.a. sequence and structural conservation. Strong a.a. sequence conservation was observed within the amino (N) terminus in all three Novirhabdovirus species: IHNV, VHSV, and SHRV. However, carboxyl (C) terminus similarity was not observed beyond a predicted α-helix structure observed in all rhabdoviral M proteins. We hypothesized that introduction of mutations within the conserved N-terminus would disrupt M protein functionality. Although less conserved at the primary a.a. sequence level, the C-terminal tail likely imparts some functionality due to its relatively well-conserved length in all viruses (Fig. 10). Stepwise a.a. deletions of the N- and/or C-termini were therefore used to assess their impact on M antitranscriptional activity. Starting with a five a.a. deletion (Δ5), M deletion mutations increased to a total of twenty a.a. (Δ20) (Fig. 11). While these significant mutations (ie deletions) are certain to affect protein function significantly, they are superior to smaller (ie single or double) point mutations that readily revert to wild type in rapidly replicating viruses such as IHNV. As such, we decided to move forward with the approach.
Figure 10. Rhabdovirus Matrix Protein Alignment. Matrix protein sequences from the following rhabdoviruses are shown: IHNV (Sequence ID: ADB93798.1), VHSV (Sequence ID: ADB93791.1), SHRV (Sequence ID: NP_050582.1), VSV (Sequence ID: NP_041714.1), and SVCV (Sequence ID: NP_116746.1). Conserved and similar residues are indicated by red shaded and blue boxes, respectively. Pink and green highlighted regions indicate predicted α-helix and β-sheet secondary structures, respectively.
Figure 11. Schematic of IHNV M deletion mutations. IHNV M deletion mutations sharing the missing residues on the N- and/or C-termini, as indicated by dotted, open boxes.
3.2.3 Functional characterization of M deletion mutants on transcriptional inhibitory activity.

Suppression of host transcription by the M deletion mutants was assessed by using the constitutively active pCAG/luc reporter. EPC and RT-Gill cells were co-transfected with pCAG/luc and three doses (0.01 µg, 0.05 µg, and 0.1 µg) of each M deletion mutant for 48 h, after which luciferase activity was quantified (Fig. 12). At these doses, WT M exhibited a dose-dependent inhibition of constitutive luciferase expression deemed, based on previous work (48), to result from inhibition of cellular transcription. By comparison, many of the individual IHNV M N- or C-terminal deletion mutants exhibited altered antitranscriptional effects. N-terminal deletion mutants of all lengths, with the exception of the Δ5N deletion, exhibited decreased inhibitory activity. N-terminal deletions greater than five a.a. showed substantial impairment, effectively nullifying their antitranscriptional activities. C-terminal deletions of less than twenty a.a. showed little change in transcriptional inhibition as compared to WT M. Interestingly, when the Δ5NC mutant (i.e., five a.a. deletions from both termini) was tested, it appeared that the presence of the added C-terminal deletion restored activity to WT levels, as compared to the Δ5N mutant alone (Fig. 12). The Δ10NC and Δ20NC mutants both exhibited attenuated, but still detectable antitranscriptional responses. However, the Δ10N20C mutant was functionally inactive, even at the highest dose tested. While some differences in mutant M antitranscriptional activity exist between the two transfected cell lines, the general results were largely consistent. Overall, these data suggest that deletions of both the N- and C-termini or
deletions of the N-terminus larger than five a.a. in the IHNV M protein result in decreased antitranscriptional activity, indicating a functional importance within the N-terminus of the M protein.
Figure 12. Impact of amino acid deletion mutations in IHNV M transcriptional inhibition. (A) EPC and (B) RT-gill cells were co-transfected with 0.5 µg or 0.8 µg pCAG/luc, respectively, plus three doses of each IHNV M deletion mutation plasmid. Closed circular parental pcDNA3.1 plasmid was used for transfection balancing and
baseline control. Luciferase activity was analyzed at 48 h post transfection and RLUs were normalized to total protein concentration in each sample, followed by normalization to the pcDNA3.1 plasmid control. Data are representative of three independent experiments performed in triplicate. Values are group means ±SEM.
3.2.4 Mutant IHNV matrix protein expression.

Immunoblot analyses was used to assess IHNV M deletion mutant expression in EPC cells. Cells were transfected with 3 µg of each M deletion mutant, in addition to WT M or empty pcDNA3.1 plasmid for 48 h. Total cell lysates were immunoblotted with antibodies to MYC to detect the tagged M protein and then β-Actin antibodies to normalize for protein loading (Fig. 13). All deletion mutants detected were expressed at their predicted sizes, albeit at varying levels. Interestingly, none of the N-terminal-only deletion mutants (Δ5N, Δ10N, and Δ20N) were well-expressed in these studies, with some barely detectable. However, when the N-terminal deletion was coupled with a concurrent C-terminal deletion, expression of these double deletion mutants was observed. These data suggest that the N-terminus of IHNV M plays a critical role in protein structure or stability, but that introduction of a C-terminal deletion compensated for this in some way and restored protein expression or stability.
Figure 13. N-terminal deletion of IHNV M causes loss of protein expression. EPC cells were transfected with 3 μg of IHNV M deletion mutant plasmids and sampled at 48 h post transfection. Cell lysates were separated by SDS-PAGE and immunoblotted for protein expression with a Myc MAb, followed by a β-Actin MAb to normalize for protein loading.
To rule out a defect in N-terminal M mutant transcription, RNA purification and RT-qPCR was performed. Cells were transfected with 0.2 µg of IHNV M WT or individual deletion mutation plasmids. After 24 h, RNA extracted from transfected cells was reverse transcribed into cDNA and IHNV M gene expression quantified using the 2-ΔΔCT method following qPCR amplification. Values were normalized to β-Actin cDNA values and compared to IHNV M WT signals (Fig. 14). At 24 h post-transfection, all IHNV M deletion mutants, with the exception of Δ20NC, were expressed at levels comparable to or greater than WT M. While protein expression was confirmed with the Δ20NC mutant, it is unclear at this time why no mRNA was detected and is considered an artifact. Three deletion mutants (Δ10N, Δ10NC, and Δ10N20C) exhibited substantially elevated levels of mRNA. These data suggest that N-terminal IHNV M deletion mutants were transcribed, but that loss of protein stability or rapid degradation prevented immunoblot detection.
Figure 14. Relative mRNA levels of IHNV M deletion mutants compared to wild-type M. RT-qPCR analysis of mRNA levels of IHNV M deletion mutants graphed as fold change normalized to WT M. Data are representative of three independent experiments performed in triplicate. Values are group means ±SEM.
3.2.5 Impact of proteasome inhibition on WT and Δ10N M deletion mutant expression.

We hypothesized that the N-terminus of IHNV M was critical for protein stability, and that its absence led to proteasomal degradation. To test this hypothesis, cells transfected with WT M, Δ10N, or Δ10NC were treated with the potent and selective proteasome inhibitor, MG132, to reduce the degradation of ubiquitin-conjugated proteins via inhibition of the 26S proteosome (73). EPC cells were individually transfected with 6 µg of empty pcDNA3.1, WT IHNV M, Δ10N mutant, or the Δ10NC mutant, and then treated with 50 µM MG132 or volume equivalent of DMSO 24 h post-transfection. 24 h later, cell lysates were incubated with Ni-NTA Agarose beads to capture 6x-HIS tagged WT and mutant M proteins, then immunoblotted sequentially with MYC and ubiquitin antibodies after elution. MG132 treatment did not restore protein detection of the Δ10N mutant, nor appreciably change detection of the other IHNV M proteins (Fig. 15). However, it did lead to accumulation of ubiquitinated proteins, demonstrating successful MG132 treatment (Fig. 15). These data suggest that an alternative, non-proteasomal protein degradation pathway may play a role in the loss of IHNV M N-terminal deletion mutant detection, or that another explanation is responsible for N-terminal M deletion protein loss.
Figure 15. IHNV M deletion mutant detection following proteasomal inhibition. EPC cells were transfected with 6 µg of empty pcDNA3.1 plasmid, IHNV M WT plasmid, and Δ10N or Δ10NC IHNV M deletion mutant plasmids. At 24 h post transfection, cells were treated with 50 µM MG132 or DMSO vehicle. Cells were sampled following 24 h treatment, 48 h post transfection. Cell lysates underwent affinity purification using Ni-
NTA Agarose beads to concentrate 6x-HIS WT and mutant M tagged proteins. Concentrated protein samples were separated by SDS-PAGE and immunoblotted for protein expression with a Myc mAb. Afterwards, blots were stripped and re-probed with a ubiquitin mAb to observe changes in protein polyubiquitination.
3.3 Conclusion

Production and functional characterization of IHNV M N- and C-terminal deletion mutations provided novel insight in the structure-function relationship of the IHNV M protein. Introduction of N-terminal deletions of five to twenty a.a., led to loss of protein detection and reduced antitranscriptional activity. However, introduction of paired C-terminal deletions greater than 5 a.a. in length resulted in protein detection stabilization and, in some cases, enhanced inhibitory activity. RT-qPCR data confirmed that all deletion mutants that exhibited loss of protein detection were expressed at the mRNA level, indicating that loss of protein expression was not related to defective transcription of M deletion mutants. Although lack of mutant detection was likely associated with protein stability or degradation, treatment of transfected cells with the proteasome inhibitor MG132 failed to restore N-terminal deletion mutant protein expression. Table 3 summarizes the changes in M transcriptional inhibitory activity and protein expression among all M deletion mutants, as compared to WT M. The 10NC and 20C M mutations were deemed worthy of further analysis, including introduction into an IHNV reverse genetics system with the goal of assessing their ability to support rIHNV replication. These 10NC and 20C mutations were highlighted based on their reduced, but not abolished inhibitory activity, and robust protein expression. Together, these data propose a functional and structural importance of the N-terminal region of the IHNV M protein, and a potentially complementary role for the C-terminus in modifying the effects of the N-terminus.
Table 3. Data summary of IHNV M deletion mutant antitranscriptional activity and protein expression

<table>
<thead>
<tr>
<th>Mutant</th>
<th>0.01 µg (A) Inhibitory Activity %</th>
<th>0.05 µg (A) Inhibitory Activity %</th>
<th>0.1 µg (A) Inhibitory Activity %</th>
<th>Overall (B) Inhibitory Activity</th>
<th>Protein Expression</th>
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</thead>
<tbody>
<tr>
<td>WT</td>
<td>67</td>
<td>81</td>
<td>85</td>
<td>+++</td>
<td>Y</td>
</tr>
<tr>
<td>5N</td>
<td>40</td>
<td>61</td>
<td>67</td>
<td>++</td>
<td>N</td>
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<tr>
<td>5C</td>
<td>74</td>
<td>85</td>
<td>90</td>
<td>+++</td>
<td>Y</td>
</tr>
<tr>
<td>5NC</td>
<td>75</td>
<td>84</td>
<td>91</td>
<td>+++</td>
<td>Y</td>
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<tr>
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<td>63</td>
<td>80</td>
<td>86</td>
<td>+++</td>
<td>Y</td>
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<tr>
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<td>5</td>
<td>27</td>
<td>21</td>
<td>+</td>
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<td>63</td>
<td>56</td>
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<td>Y</td>
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<tr>
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<td>22</td>
<td>+</td>
<td>Y</td>
</tr>
<tr>
<td>10N20C</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>Y</td>
</tr>
</tbody>
</table>

(A) Inhibitory values represent averages from at least three independent experiments performed in triplicate.

(B) Overall inhibitory activity was defined as high or ≥ 75% (+++), medium or ≥ 50% (++), low or ≥ 20% (+), and abolished or ≤ 10% (-) at the highest (0.1 µg) transfection dose.

(*) Mutants with desired anti-host characteristics and robust protein expression that were selected for further analysis.
Chapter 4: DEVELOPMENT AND RECOVERY OF AN INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS REVERSE GENETICS SYSTEM

4.1 Rationale

A reverse genetics system for IHNV was first developed over two decades ago utilizing a T7 RNA polymerase strategy to drive viral RNA genome transcription (64). Subsequent strategies placed the reverse genetics system under the control of a CMV promoter to simplify and improve the recovery of recombinant negative strand RNA viruses (65). In order to fully study the impact of attenuated IHNV M deletion mutants and their reduced inhibitory effects on host immune responses and their ability to support recombinant virus replication, a modified reverse genetic system must be developed to permit efficient introduction of various M deletion mutants into the viral genome. Such a system is critical for the rapid manipulation and incorporation of various IHNV genes, thereby allowing for further assessment of their physiological impact on replication, innate immune system activation and anti-host activities.

In addition to the optimization of genetic manipulation, development of a novel IHNV reverse genetics system also fuels the development of a live-attenuated recombinant virus for use as a potential vaccine candidate. For over thirty years, researchers have attempted to develop IHNV vaccines utilizing a variety of techniques: recombinant G protein inoculation (74), introduction of neutralization-resistant mutants (75), and use of recombinant IHNV that contained a rearranged gene order (76). Unfortunately, no effective treatment for IHNV infected fishes has advanced to commercial application, further driving
the need for an efficient and safe vaccine target. Here, we aimed to engineer a novel IHNV reverse genetics system that allowed for straightforward introduction of characterized mutants within the M or NV viral genes and robust recovery of a recombinant mutant virus.

4.2 Results

4.2.1 Construction of a full-length cDNA clone of the IHNV genome that allows for efficient introduction of matrix and nonvirion gene mutations.

In order to develop a novel IHNV reverse genetics system that allows for efficient integration of mutations within the M (and NV) gene, a full-length clone of IHNV 220-90 (GenBank: GQ413939.1) was assembled in an unmodified pCI vector (Fig. 16). To promote viral recovery, self-cleaving ribozyme sequences (HHRZ and HdvR) were fused at both cDNA ends, allowing for precise RNA cleavage, leaving more authentic terminal viral RNA ends. Unique restriction sites were introduced on either side of the coding regions of the M (KpnI/MluI) and NV (SphI/SacII) genes to facilitate rapid introduction of modified/characterized mutant genes. The modified viral genome was synthesized and assembled into the pCI vector commercially (GENEWIZ South Plainfield, NJ, USA).
Figure 16. IHNV reverse genetics system schematic and recombinant virus cloning strategy. Schematic of a full-length cDNA clone of IHNV 220-90 within a cytomegalovirus (CMV) controlled pCI vector that includes hammerhead ribozyme (HHRz) and hepatitis delta virus ribozyme (HdvR) cDNA sequences were fused at the 5’ and 3’ ends, respectfully. Restriction sites flanking the coding regions of both M (KpnI/MluI) and NV genes (SpeI/SacII) are highlighted.
4.2.2 Observation of cytopathic effects and rescue of recombinant IHNV from cDNA template.

To generate viable recombinant IHNV (rIHNV), EPC cell monolayers were transfected in six-well plates with a mixture of the full-length cDNA IHNV clone within the pCI vector and supporting pCI plasmids containing various unmodified IHNV genes (N, P, and L) (Fig. 17). In order to optimize transfection conditions for rIHNV recovery, several transfections were performed with different amount and combinations of the full-length genome and supporting plasmids (Fig. 18a). Two different IHNV NV supporting pcDNA3.1 plasmids were utilized, one containing in-frame MYC and polyhistidine tags (NV-T), and another without tags (NV). Following transfection, cytopathic effects (CPE) were observed as early as 3 days post-transfection. At 4 days post-transfection, supernatants were collected and stored at -80°C for future use and replaced with complete media. CPE continued to progress until complete monolayer death was observed in some wells, around 9 days post-transfection (Fig. 18b). The results indicate that a combination including 1 µg of full-length plasmid, 0.5 µg of pCI-N, 0.2 µg of pCI-P, 0.2 µg of pCI-L, and 0.15 µg of either pcDNA3.1-NV plasmids (NV-T/NV) was the most optimal for strong CPE induction. Transfection conditions favoring robust levels of CPE were repeated to ensure cytopathic consistency, and total cell lysates were collected at 5 days post-transfection stored at -80°C for future use. Together, these results suggest that recombinant IHNV was produced through successful engineering of a complete reverse genetics system. Whether the rIHNV is infectious or not remains to be determined.
In order to produce recombinant IHNV via a reverse genetics system, EPC cells were transfected with a combination of the full-length rIHNV-pCI plasmid along with combinations of N, P, and L-pCI support plasmids. MYC and 6xHis-tagged Wild-type NV (indicated as NV-T) or untagged wild-type NV within pcDNA3.1 (indicated as NV) was also co-transfected within these combinations. Following transfection, the onset of cytopathic effects such as cell lysis and plaque formation was assessed as a surrogate indicator of virus production.
Figure 18. Observed cytopathic effects induced by recombinant IHNV component transfection. (A) EPC cells were transfected at room temperature with various
combinations and concentrations of full-length rIHNV-pCI, N-pCI, P-pCI, L-pCI, and tagged or untagged NV-pcDNA3.1 (Tagged indicated as NV-T) support plasmids. Following transfection, cells were maintained at 20°C until cytopathic effects were observed. (B) EPC cells were left untransfected or were transfected as indicated in (A). After 9 days of transfection, cell images were captured at 400x magnification under phase contrast. Maximal CPE was observed only when all components illustrated in (A) were included.
4.3 Conclusion

Transfection of EPC cells with full-length-IHNV pCI in combination with pCI-N, -P, and -L and pcDNA3.1-NV (tagged or untagged), yielded significant CPE by 9 days post transfection. Other transfection combinations that omitted one or more of these plasmid components exhibited little to no CPE. These data suggest that the rIHNV system is functioning in a manner similar to previous efforts by other groups, although verification will require isolation and propagation of infectious rIHNV viruses, which was beyond the scope of this work.
Chapter 5: DISCUSSION

Although discovered over seventy years ago, IHNV remains a dangerous pathogen that impacts the global aquaculture industry and aquatic ecosystems worldwide. Approaches to vaccine development against IHNV have shown moderate success, but have not yet achieved commercial viability. Therefore, the development of a novel strategy to achieve protective immunity against IHNV is critical for addressing this significant pathogen threatening our seafood supply. The overall objective of this study was to develop a novel, attenuated recombinant IHNV (rIHNV) that can be used as a potential vaccine candidate. Our central hypothesis is that a rIHNV that is impaired in its ability to suppress host responses and, perhaps, replicate more slowly will activate an immune response that leads to protective immunity. To address our hypothesis, one approach undertaken was to introduce an array of mutations within the IHNV M gene to reduce host antitranscriptional effects but maintain protein stability and some pro-viral functions. Previous research from our lab had shown that attenuation of recombinant VHSV through the introduction of point mutations within the M gene yielded such features (48). However, as shown in these studies, compatible mutations did not produce similar effects within the IHNV M gene (Fig. 9). Instead, introduction of IHNV M end terminal deletion mutations, particularly those that targeted both ends simultaneously, exhibited reduction, or complete loss, of antitranscriptional activity. Furthermore, our data suggested that the N-terminal region of IHNV M is critical for protein stability, either directly or indirectly. The fact that expression was rescued with the introduction of an additional C-terminal deletion argued
for a structural requirement that impacted stability. Inhibition of proteasomal degradation pathways with MG132 treatment did not rescue protein detection or stability in N-terminal deletion mutants larger than 5 a.a., indicating alternative degradation pathways may play a role.

While *Novirhabdovirus* M protein cellular localization and functions have been studied (48, 77), very little is known about M’s 3-dimensional structure. Several studies have provided insight into the structure of VSV matrix protein, but not VHSV or IHNV (78, 79). In those cases, N- and C-terminal structures was disordered, making it difficult to predict how deletion mutations to either end of the IHNV matrix protein might alter structure. While we were expecting potential loss of structural stability of some of our M mutants, we were surprised to see the expression of N-terminal mutants restored by introduction of an additional deletion at the C-terminus. This phenomenon was observed not just with five a.a. C-terminal deletions, but also with larger deletions of up to twenty a.a. in size. Although not detailed in these regions, the VSV M crystal structure suggested that it is possible that both termini interact in their native state (78, 79), providing structural integrity. However, upon deletion of the N-terminus, that interaction is disrupted, leading to protein instability and degradation (Fig. 19). When both termini are deleted, it is possible that new terminal interaction sites develop or simply less tension due to increased freedom of motion allows for recovery of protein stability. Alternatively, deletion of N-terminal a.a.
Figure 19. Proposed model of IHNV M terminal interactions in WT and deletion mutants. Three separate M protein structure conformations are presented and include a proposed ubiquitin or other targeted degradation site (indicated as red circle) and labeled N- and C-termini, respectfully. We hypothesize that WT M contains an inaccessible degradation site (or signal), masked by interactions between the wild type N- and C-termini. Upon deletion on the N-terminus, a conformational change in protein structure exposes the previously inaccessible degradation site, leading to protein instability and/or degradation. Subsequent C-terminal deletion mutations compensate for this by removing the newly accessible degradation site, restoring protein stability but not necessarily full protein function, depending on the size of the deletions.
may also alter protein structure in such a way to promote protein degradation, perhaps by uncovering formerly inaccessible C-terminal ubiquitination sites that enhance degradation. Introduction of C-terminal deletions may remove these sites and thus reduce targeted degradation. Attempts to test this possibility were performed by assessing the effect of select proteasome inhibitors, to see if they impacted mutant matrix protein expression. MG132 was unable to restore mutant M expression (Fig. 15), but it is possible that other proteasomal inhibitors such as Bortezomib, Lactacystin, and CEP1612 could yield more definitive results. Alternative protein degradation pathways may play a role in N-terminal deletion mutant non-detection, such as lysosomal degradation. A plethora of lysosomal and autophagy inhibitors are available, making this an excellent path for future study.

Our studies led us to propose two prime candidates for further study, the Δ10NC and Δ20C mutants. These mutants both exhibited reduced transcription inhibition but retained robust protein expression. Deletion mutations are less targeted than a.a. point mutations, but mutation reversion is nearly impossible, allowing for long term propagation of mutant strains. This feature is critical in the development of an attenuated recombinant virus that provides protective immunization, as previously reported IHNV vaccine strategies often suffer from genetic reversion or loss of long-term mutant propagation (3).

The second goal of this study was to develop a novel IHNV reverse genetics system that allowed for straightforward introduction of characterized mutations within IHNV. Although our data provide evidence that our modified IHNV reverse genetics system produced recombinant virus that exhibits cytopathic effects, additional work is needed to
verify this. While previous studies demonstrated modified genomes are tolerated when used with a T7 RNA polymerase (64) or a vaccinia-virus free system (65), this is the first IHNV system that allows for straightforward viral genome modification through the introduction of specific restriction sites that allow rapid insertion/removal of the M or NV genes. As reported in those prior studies (65), further analysis of recombinant IHNV characteristics through RT-PCR detection, propagation, and stock preparation, as outlined below.

5.1 Future directions

Future experiments should focus on further development of the proposed attenuated recombinant IHNV. In particular, this will involve creation of a virus containing our M mutants, including Δ10NC and Δ20C. Those studies will be critical for determining whether either of these mutants can support viral production and replication. Beyond that, future work must also be done to fully characterize the newly engineered mutant recombinant IHNV. Studies should focus on establishing viral titers via RT-qPCR and plaque assay assessment. In addition, viral growth kinetics and IFN bioassays should be performed in order to establish impact on host transcription, comparative pathogenicity, and inhibition of host-IFN antiviral responses compared to WT IHNV.

Lastly, with the discovery of the critical role of the IHNV M N-terminus in regulating host expression and protein stability, future studies could focus on building a deeper understanding of its structural relevance within the IHNV M protein sequence. Specifically, experiments should work to uncover the relationship between N-terminal
deletions and protein detection following additional C-terminal deletion. Work investigating different proteasomal and lysosomal protein degradation pathways in order to rescue N-terminal deletion mutant instability should occur. Alternatively, studies may be conducted in order to further understand N-terminal deletion mutant conformational changes and their relationship with polyubiquitination sites within IHNV M.

5.2 Conclusions

Collectively, these findings suggest the N-terminal and C-terminal regions of IHNV M exhibit functional importance in regulating host transcriptional suppression and play critical roles in overall protein structure or stability. Mutations that exhibit reduced antitranscriptional activity and are robustly expressed make for excellent candidates in future studies as a means of developing protective immunization with a novel attenuated recombinant IHNV.
References


77. Chiou PP, Kim CH, Ormonde P, Leong JA. 2000. Infectious hematopoietic necrosis virus matrix protein inhibits host-directed gene expression and


1. Introduction

Fish Rhabdoviruses are responsible for causing fatal epizootics within commercial and wild populations of various fish species around the world. Infectious hematopoietic necrosis virus (IHNV), also known as the Salmonid novirhabdovirus, is enzootic along the Pacific Coast of North America and is comprised of three genogroups, each of which is endemic to a specific geographical location (1). Once the virus enters the host through the fin epithelia, IHNV infection causes infectious hematopoietic necrosis in salmonid species. The disease is highly fatal and presents with signs such as abdominal distension, bulging of the eyes, anemia, and necrosis of vital organs such as the liver and kidneys, all caused by systemic hemorrhaging within the host.

The 11-kb negative-sense, ssRNA viral genome within IHNV consists of six genes that encode the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), nonvirion protein (NV), and RNA-dependent RNA polymerase (L), in order from 3’ to 5’, respectively (2). While most of the protein products from the IHNV genome have been studied and elucidated, the precise functions of the NV protein remain unknown. While multiple studies have reported various roles for NV, such as suppression of apoptosis, interferon (IFN) induction, and NF-κB activation (3,4,5). Data from our lab suggest that NV augments host expression of a constitutively active luciferase reporter gene (6) and/or
plays a direct role in the PERK-eIF2α pathway, shutting off host translation in infected cells through increased eIF2α phosphorylation (7). Regardless of the proposed functions of NV, functional sites within the viral protein are poorly defined. Here we aim to investigate the structure-function relationships of various regions within the IHNV NV protein as a means of identifying regions that contribute to NV’s anti-host functions in order to develop an attenuated recombinant virus. We hypothesize that IHNV NV terminal deletion mutants will yield an IHNV NV mutant with the desired attenuated activity for vaccine studies. In order to test our hypothesis, focus was placed on the introduction of C- and N-terminal deletion mutations (ΔNV). Data within the body of this thesis has shown successful attenuation of IHNV M protein anti-host responses, therefore we predict that introduction of similar deletion mutations within IHNV NV will lead to similar reduction of transcriptional/translational modulation.
2. Materials and Methods

2.1 Cell lines and culture maintenance

Epithelial cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA), including Fathead minnow (*Pimephales promelas*) *Epithelioma papulosum cyprini* (EPC) (ATCC: CRL-2872), and Rainbow trout (*Oncorhynchus mykiss*) gill (RTgill-W1) (ATCC: CRL-2523). Cell cultures were maintained in 25 cm² tissue culture flasks (CytoOne, Ocala, FL, USA) at 20°C, with L-15 Leibovitz media (HyClone, Marlborough, MA, USA) supplemented respectively with 1% Penicillin-Streptomycin (PS) solution (Corning, Corning, NY, USA), and with 2% (L15-2PS) or 10% (L15–10PS) fetal bovine serum (Corning, Corning, NY, USA) without CO₂. Complete media were sterile-filtered through a 0.2 μm cellulose nitrate membrane (Nalgene, Rochester, NY, USA).

2.2 IHNV plasmids and luciferase reporters

The IHNV NV gene from M genogroup were cloned into expression plasmids for transient co-transfections experiments as previously described (6). Coding sequences were PCR-amplified using cDNA from archive viral stocks with specific primers (Table 1) to generate mutations within the IHNV NV gene. PCR fragments were cloned into EcoRI and KpnI sites of pcDNA3.1(−)Myc/His A plasmid (Invitrogen, Waltham, MA, USA). Plasmids were amplified in *E. coli* DH5α cells and plasmid DNA was purified using Qiagen Plasmid Plus Midi Kit (Qiagen, Hilden, Germany), following the manufacturer’s instructions. All expression plasmids were sequence confirmed before
## Supplemental Table 1. Primers for Cloning

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’ → 3’)</th>
<th>Restriction Site</th>
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<td>IHNV NV se</td>
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<td>EcoRI</td>
<td></td>
</tr>
<tr>
<td>IHNV NV as</td>
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<td>IHNV NV Δ5N se</td>
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</table>
use in transfection experiments. The construction of other expression plasmids was previously reported (8). A luciferase reporter construct harboring the CMV enhancer/chicken β-Actin promoter, pCAG/luc plasmid (9), was purchased from Addgene (Plasmid #55764).

2.3 Transient Cell Transfection

Transfections were performed in L15–10, without addition of antibiotics, using the suitable transfection reagent for each cell line at a final 3:1 reagent volume (µl) to total DNA amount (µg). EPC cells were transfected using FuGENE HD Transfection Reagent (Promega, Madison, WI, USA), while RTgill-W1 were transfected using Via-Fect Transfection Reagent (Promega, Madison, WI, USA). Plasmid concentrations in all transfection experiments were equalized between samples by the inclusion of empty vector pcDNA3.1, which was also used in negative control groups. DNA mixtures were complexed with the respective transfection reagents in 37°C pre-warmed Opti-MEM I reduced serum medium (Gibco, Waltham, MA, USA), then incubated for 15 min at room temperature. 100 µl transfection doses were added to each confluent cell monolayer with 500 µl L15–10 in each well. 12-well plates (CytoOne, Ocala, FL, USA) were incubated at 20°C, without any further medium replacement or manipulation until the indicated sampling points.

2.4 Luciferase reporter assay

Luciferase assays were performed as described by Gorgoglione, et al. (6). Briefly, after the designated time post-transfection, cell monolayers in each well were gently
washed with 1X PBS at 20 °C, then lysed for 15 min at RT in 200 μl of luciferase cell culture lysis reagent (Promega, Madison, WI, USA), used at 1X in molecular grade water (HyClone, Marlborough, MA, USA). Sampled 12-well plates were used immediately or kept at −20°C until assays were performed. From each cell lysate sample, 75 μl was collected for Luciferase reporter assay in 96-well white solid flat bottom opaque microplates (Greiner Bio-One, Kremsmünster, Austria), and 5 μl for Bradford assay in 96-well clear flat bottom microplates (Greiner Bio-One, Kremsmünster, Austria). The luciferase reporter assay was performed by adding to each sample 100 μl of a mixture containing: 51 μl of Luciferase assay ATP assay buffer [3.83mM EGTA (MP Biomedicals), 14.4mM Magnesium sulphate (Thermo Fisher Scientific, Waltham, MA, USA), 23.9mM Glycylglycine (Thermo Fisher Scientific, Waltham, MA, USA), 14.4mM Potassium phosphate dibasic (Thermo Fisher Scientific, Waltham, MA, USA), 0.98mM DTT (Dithiothreitol, Thermo Fisher Scientific, Waltham, MA, USA), 1.97mM ATP (Thermo Fisher Scientific, Waltham, MA, USA 0.33mM Coenzyme-A (Thermo Fisher Scientific, Waltham, MA, USA), in Milli-Q water], and 49 μl of Luciferin solution [1mM DTT, 25.1mM Glycylglycine, 0.27mM D-Luciferin (Thermo Fisher Scientific, Waltham, MA, USA), in autoclaved Milli-Q water]. Luminescence light emission was measured with a Synergy H1 microplate reader (BioTek Instruments, Winooski, VT, USA), setting top optics reading and luminescence spectral scanning gain/sensitivity to 135. The total protein load was measured by adding 5 μl of each cell lysate to 95 μl of Pierce™ Coomassie Plus (Bradford) solution (Thermo Fisher Scientific, Waltham, MA, USA) (diluted 50% in
autoclaved Milli-Q water). Light absorbance values were immediately read at 595 nm with a Synergy H1 microplate reader. Luminescence data, expressed as Relative Light Units (RLU), were normalized to lysate protein concentrations. The Relative Luciferase Activity (RLA) was calculated as the % ratio between stimulated (co-transfected with testing plasmids) and unstimulated (pcDNA3.1 alone) samples. All data shown are representatives of at least three independent experiments and presented as group means (±SEM).

2.5 Immunoblotting

The expression of viral plasmids transfected in fish cells was assessed by Western blotting. Cell lysates were prepared and separated by SDS-polyacrylamide gel electrophoresis (PAGE), as previously described (7). Samples were electrophoretically transferred to Immobilon®-P PVDF membrane (MilliporeSigma, Burlington, MA, USA) and membranes were blocked with 5% (w/v) BSA/TBST (P-753, Boston Bioproducts, Ashland, MA, USA) for 1 h at room temperature. Primary antibodies, anti-Myc monoclonal (Myc, Invitrogen, Waltham, MA, USA), anti-β-Actin (Thermo Fisher Scientific, Waltham, MA, USA), or anti-Ubiquitin polyclonal (Invitrogen, Waltham, MA, USA) were diluted in TBST at 1:5000 and incubated overnight at 4°C. Membranes were incubated with the secondary antibody for 1 h at RT, using horseradish peroxidase (HRP)-conjugated Goat anti-mouse IgG1 (Invitrogen, Waltham, MA, USA) or anti-rabbit IgG, HRP-linked antibody #7074 (Cell Signaling Technology, Danvers, MA, USA) at 1:10,000 or 1:5000 dilution in TBST, respectively. Immunoreactive bands were visualized with SuperSignal™ West Pico PLUS chemiluminescent substrate (Thermo Fisher Scientific,
Waltham, MA, USA) using Amersham Imager 600 (General Electric, Boston, MA, USA). Following exposure with anti-Myc or anti-Ubiquitin antibodies, membranes were stripped using Restore™ PLUS western blot stripping buffer (Thermo Fisher Scientific, Waltham, MA, USA) for 10 min at room temperature, thereafter, blocked with 5% (w/v) BSA/TBST for 30 min at room temperature, and re-probed with the anti β-Actin antibody.
3. Results

Stepwise amino acid deletions of the N- and/or C-termini were used to assess their impact on the ability of NV to upregulate constitutive gene or protein expression via co-transfection studies with a luciferase reporter. Starting with a five amino acid deletion (Δ5), NV deletion mutations increased to a total of twenty amino acids (Δ20) (Suppl. Fig. 1). While these significant mutations (i.e. deletions) are certain to affect protein function significantly, they are superior to smaller (i.e. single or double) point mutations that revert to wild type in rapidly replicating viruses such as IHNV. Suppression of host transcriptional/translational modulation by the NV deletion mutants as compared to WT NV was assessed by using the constitutively active pCAG/luc reporter. EPC and RT-Gill cells were co-transfected with pCAG/luc and one dose (0.1 µg) of each NV deletion mutant for 48 h, after which luciferase activity was quantified (Supplemental Fig. 2). In both cell lines, WT NV exhibited robust transcription/translational augmentation as measured by increased luciferase activity. By comparison, many of the IHNV NV N- or C-terminal deletion mutants exhibited altered (mostly reduced) modulatory effects (Suppl. Fig. 2). Introduction of N-terminal deletions had little effect on transcription/translational augmentation as compared to WT NV. However, C-terminal deletions of all sizes, with the exception of Δ5C in RT-Gill cells, exhibited decreased ability to modulation transcription/translation. Introduction of an additional C-terminal mutation of nearly any size effectively nullified transcriptional/translational modulatory activities, particularly in
the EPC transfected Δ5N10C mutant. While differences in mutant NV activity was observed between the two transfected cell lines, the overall results are largely consistent.

Immunoblot analyses were used to assess IHNV NV deletion mutant expression in EPC cells. Cells were transfected with 3 µg of each NV deletion mutant, in addition to WT M, or empty pcDNA3.1 plasmid for 48 h, and total cell lysates immunoblotted with antibodies to MYC to detect the tagged M protein and then β-Actin antibodies to normalize for protein loading (Suppl. Fig. 3). All deletion mutants were expressed at the predicted size, albeit at varying levels. Despite this variability, the data suggest that changes in protein expression alone were not responsible for the reduced activities of some mutants that exhibited impaired modulation of basal transcription/translation. Overall, these data suggested that deletions of both the N- and C-termini or deletions of just the C-terminus in the IHNV NV protein resulted in decreased transcriptional/translational modulation, indicating functional and/or structural importance of the C-terminus of the NV protein.
Supplemental Figure 1. Schematic of IHNV NV deletion mutations. IHNV NV deletion mutations are indicative of missing amino residues on N- and/or C-termini, correlative to their deletion size, indicated by dotted box.
Supplemental Figure 2. Impact of amino acid deletion mutations in IHNV NV transcriptional inhibition. (A) EPC and (B) RT-gill cells were co-transfected with 0.5 µg
or 0.8 µg pCAG/luc, respectively, plus 0.1 µg of each IHNV NV deletion mutation plasmid. Circular empty pcDNA3.1 plasmid vector was used for transfection balancing and baseline control. Luciferase activity was analyzed at 48 h post transfection and RLUs were normalized to total protein concentration in each sample, followed by normalization to pcDNA3.1 plasmid control. Data are representative of three independent experiments performed in triplicate. Values are group means ±SEM.
Supplemental Figure 3. Protein expression of IHNV NV deletion mutants. EPC cells were transfected with 3 µg of IHNV NV deletion mutant plasmids and sampled at 48 h post transfection. Cell lysates were separated by SDS-PAGE and immunoblotted for transfected protein expression with a Myc MAb. Blots were striped and reprobed with a β-actin antibody to control for total lysate loading.
4. Conclusions and future directions

We hypothesized that IHNV NV terminal deletion mutants would yield an IHNV NV mutant with attenuated activity that could be cloned into our rIHNV system as part of our vaccine studies. To address our hypothesis, we introduced an array of mutations within the IHNV NV gene and assessed them for reduced host transcription/translational modulation in transient co-transfection assays. As shown in our preliminary studies here, introduction of IHNV NV end terminal deletion mutations, particularly those that targeted both ends simultaneously, exhibited reduction, or complete loss, of transcriptional modulation.

Future experiments should focus on further characterization of IHNV NV deletion mutants to determine if any of the other proposed functions of NV are similarly impacted. In particular, studies should assess whether NV deletion mutants regulate eIF2α phosphorylation abnormally as compared to WT NV. Those studies will be critical for determining more specific structure-function relationships in host translation inhibition by NV, and may even identify whether the impact on transcription/translation is a direct result of eIF2a regulation, or some additional function. It may even help uncover the mechanism of increased eIF2α phosphorylation with NV. Beyond that, future work should focus on cloning characterized IHNV NV deletion mutants that exhibit reduced anti-host properties into our proposed novel reverse genetic system, allowing for the creation of a virus containing NV mutants alone, M mutants alone, of rIHNV with both M and NV mutants, as we proposed in our original application for USDA/ARS funding.
Supplemental References


