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**THE MOLECULAR CHARACTERIZATION OF NIV C PROTEIN AND
THE IDENTIFICATION OF HENIPAVIRUS-HOST PROTEIN
INTERACTIONS**

A Dissertation in

Pathobiology

by

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Abstract

Since the emergence of the highly pathogenic *Henipaviruses* in 1990's, hundreds of human fatalities have been reported throughout Asia and Australia. Like other paramyxoviruses, *Henipaviruses* are enveloped viruses; successful replication requires the cooperation of numerous virus and host factors. One of these factors, Nipah virus (NiV) C protein, was investigated in the current research to characterize some of the molecular characteristics C protein may require for its role in viral pathogenesis and immune evasion.

Many paramyxoviruses express multifunctional C proteins; potential roles of C proteins during virus infection include: immune evasion, regulation of viral polymerase function, viral pathogenesis, and recruitment of host factors for virus particle assembly. Here, it was observed that NiV C protein is a membrane-bound protein that is also highly unstable when expressed in mammalian cells. NiV C protein turnover was also found to be ubiquitin-proteasome dependent. It was confirmed that NiV C protein is ubiquitinated and the elimination of all the sites for ubiquitin attachment was required to prevent ubiquitination and partially restore protein stability. These findings have defined new characteristics of NiV C protein, which are likely important during viral infections.

In an effort to identify proteins important for *Henipavirus* assembly and budding, several screens were performed to identify host protein interactions with *Henipavirus* matrix (M) proteins. Several novel host factors that interact with *Henipavirus* M proteins were identified. In addition, minimal binding fragments were constructed that, when overexpressed, inhibited the production of Nipah virus-like particles. A more comprehensive analysis of the identified host proteins revealed that they were frequently found in known mammalian cellular complexes with each other. These complexes are involved in a variety of cellular functions such as transcriptional regulation, protein translation, protein sorting and transport. Any number of these may be critical for

Henipavirus M protein function. Additionally, more than half of the confirmed *Henipavirus* M-interacting proteins contained large serine-rich acidic regions. These regions were critical for binding and likely allow for electrostatic interactions to occur with *Henipavirus* M proteins. Together, these data provide a model where *Henipavirus* M protein uses electrostatic interactions with host factors for a variety of functions.

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List of Abbreviations

AD – Activation domain

AMOTL1 – Angiomotin like 1

AP3 β 1 – Adaptor protein complex AP-3 subunit beta 1

ARL6IP4 – ADP-ribosylation-like factor 6 interacting protein 4

Cav-1 – Caveolin 1

CSNK2A1 – Casein kinase II subunit alpha

CUL3 – Cullin 3

DBD – DNA-binding domain

EBOV – Ebola virus

EGFP – Enhanced green fluorescence protein

EIAV – Equine infectious anemia virus

eIF3B/C – Eukaryotic translation initiation factor 3 subunit B/C

ESCRT – Endosomal sorting complex required for transport

F – Fusion

FIBP – Fibroblast growth factor (acidic) intracellular binding protein

G – Glycoprotein

H/HA – Hemagglutinin

HA-Ub – HA tagged ubiquitin

HA-Ub KO – HA tagged ubiquitin lysine knockout

HeV – Hendra virus

HIV-1 – Human immunodeficiency virus type 1

HN – Hemagglutinin neuraminidase

hPIV1-4 – Human parainfluenzavirus type 1-4

HPV – Human papillomavirus

HRSV – Human respiratory syncytial virus

HSPA8 – Heat shock 70kDa protein 8

HSV – Herpes simplex virus

IFN – Interferon

KBTBD7 – Kelch repeat and BTB domain-containing protein 7

L – Large

M – Matrix

MeV – Measles virus

MMR – Measles, mumps, rubella

MuV – Mumps virus

N/NP – Nucleocapsid

NCL - Nucleolin

NDV – Newcastle disease virus

NiV – Nipah virus

P – Phosphoprotein

PARP1 – Poly (ADP-ribose) polymerase 1

PIV5 – Parainfluenza virus type 5

RBBP6 – Retinoblastoma binding protein 6

RdRP – RNA-dependent RNA polymerase

RNP – Ribonucleoprotein

SeV – Sendai virus

STAT – Signal transducer and activator of transcription

TCOF1 – Treacher Collins-Franceschetti Syndrome 1

TEM – Transmission electron microscopy

TSG101 – Tumor susceptibility gene 101

VLP – Virus-like particles

VPS – Yeast vacuolar protein sorting

VSV – Vesicular stomatitis virus

Y2H – Yeast two-hybrid

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“It is good to have an end to journey toward; but it is the journey that matters, in the end.”

– Ernest Hemingway

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Chapter 1

Introduction

1.1 Paramyxovirus Classification and Impact

Paramyxoviruses represent a large group of enveloped negative-sense RNA viruses, which include a large number of human and animal pathogens. The *Paramyxoviridae* family is comprised of two subfamilies: *Paramyxovirinae* and *Pneumovirinae*. The *Paramyxovirinae* subfamily contains five genera: *Rubulavirus*, *Respirovirus*, *Avulavirus*, *Morbillivirus*, and *Henipavirus*. The *Pneumovirinae* subfamily only contains two genera—*Pneumovirus* and *Metapneumovirus*. The viruses contained within each genus are classified based on the organization of their genomes, sequence similarity of the encoded proteins, biological activities of these proteins, and the morphological characteristics of virus particles (Fig. 1-1). There are several notable human pathogens in the *Paramyxoviridae* family: measles virus (MeV), mumps virus (MuV), human parainfluenza virus types 1-4 (hPIV1-4), human respiratory syncytial virus (HRSV), Nipah virus (NiV), and Hendra virus (HeV) (83). Other historically significant paramyxoviruses that have impacted the agricultural industry include Newcastle disease virus (NDV) and Rinderpest virus.

Although the measles, mumps, and rubella (MMR) vaccine in developed countries largely controls MeV and MuV infections, they still persist as common childhood illnesses globally. As recently as 2006 and 2009, there have been outbreaks of MeV and MuV in the United Kingdom, Canada and the United States (5, 12, 13, 26, 122). The number of reported cases of mumps in the United States during 2006 spiked to 6,584 individuals (26). This dramatic increase in MuV infections likely occurred as a result of individuals with either waning immunity or incomplete vaccination living in close proximity to each other. Additionally, international travel also poses increased risk to

those without complete vaccination since 43% of the world's nations do not have any MMR vaccination programs (166). The lack of a global vaccination programs has resulted in over 20 million MeV infections that led to 139,300 MeV related deaths in 2010 (167)

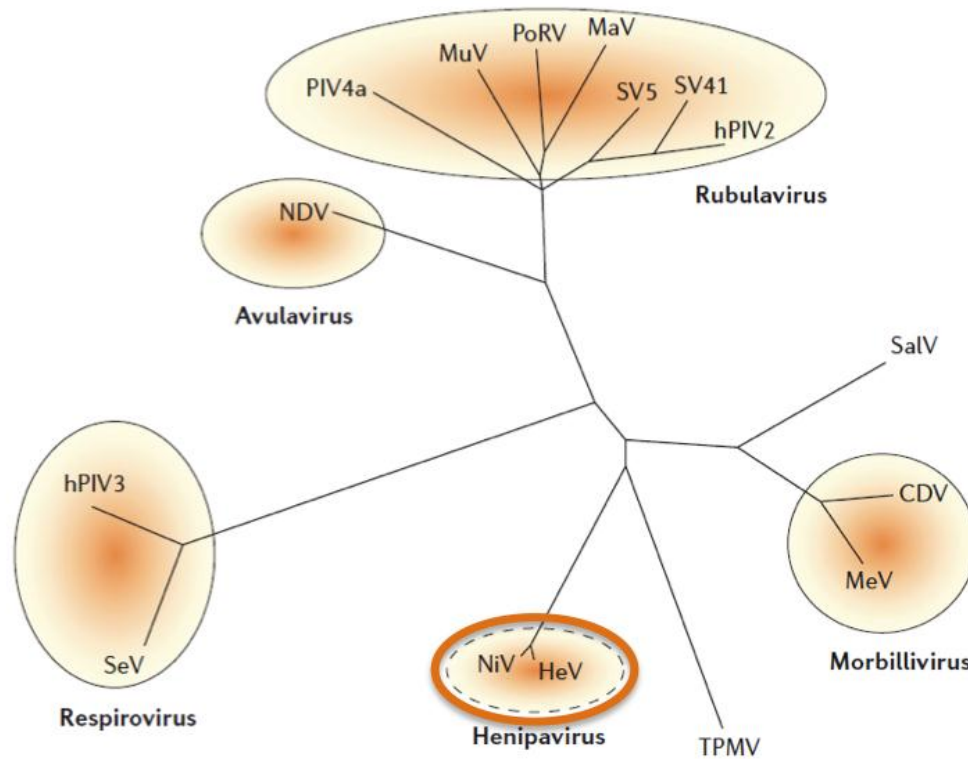
The metapneumoviruses, HRSV and hPIV types 1-4, are major contributors to respiratory infection in the elderly, young children, and individuals with suppressed immune systems (164). Currently, there are no vaccines to prevent HRSV and hPIV1-4 infections in these demographics, and as a result, severe infections are commonly treated therapeutically with the antiviral drug ribavirin (53, 165). Individuals infected with HRSV or hPIV1-4 also do not develop any long lasting immunity. Consequently, both viruses can repeatedly infect individuals causing lower respiratory tract infections throughout an individual's lifetime.

Two recently-emerged highly pathogenic paramyxoviruses, NiV and HeV, have had a widespread impact on humans and the agricultural industry in Southeast Asia and Australia (35). Since their emergence in the 1990's NiV and HeV have been responsible for over 200 human fatalities throughout Asia and Australia (Reviewed in 35).

Henipaviruses are zoonotic viruses that normally reside in a natural host reservoir, the pteropid fruit bat. Occasionally, virus spill over events have led to infections in horses, pigs, and humans resulting in high mortality of these secondary hosts. For NiV, initial cases were thought to be Japanese encephalitis. However, when subsequent vaccination efforts were ineffective, the virus was identified as a new paramyxovirus closely related to HeV, which had been previously identified in 1994 (18, 169). These initial reports of NiV infection occurred concomitantly with a widespread infection in pigs, and human infection was a result of close direct contact with infected animals (115). Although in more recent outbreaks in Bangladesh, NiV transmission was found to be

bimodal. NiV infections occurred from both human-to-human transmission and from direct human contact with virus-contaminated bat saliva, urine, or feces (52).

The typical symptoms of NiV infection include: fever, headache, confusion, and loss of consciousness potentially leading to death via vasculitis and encephalitis. One of the primary mechanisms of action in which NiV and HeV exert their pathologic effect is through the formation of syncytia. Syncytia formation is typical among most paramyxoviruses however; the NiV attachment glycoprotein (G) binds to a unique cell surface protein among paramyxoviruses, Ephrin B2/B3 (110). Ephrin B2/B3 is well conserved in many mammals and is expressed on endothelial cells, neurons, and smooth muscle cells that surround arteries and arterioles (7). Additional insight into the mode of systemic spread has demonstrated that NiV is able to be transported by peripheral blood lymphocytes and macrophages in order to allow for dissemination throughout the body and even across the blood brain barrier (99). These characteristics allow *Henipaviruses* to infect a large range of hosts and tissue types resulting in systemic infections unlike most other respiratory viruses (39, 169). Without any effective antivirals or therapeutics against these viruses, they will likely continue to threaten these areas. The focus of analyses and discussion for this dissertation will center on *Henipaviruses*.



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Phylogenetic tree of paramyxoviruses

Fig. 1-1 Phylogenetic tree of the *Paramyxovirinae* subfamily.

A phylogenetic tree of the Paramyxovirinae subfamily of the Paramyxoviridae family. The viruses shown here were categorized by a sequence alignment of the N gene product using the Neighbor-Joining method. The following viruses are grouped based on genus. *Henipavirus* genus, highlighted here for the purpose of these studies: Nipah virus (NiV); Hendra virus (HeV). *Respirovirus* genus: Sendai virus (SeV); human parainfluenza virus type 3 (hPIV3). *Avulavirus* genus: Newcastle disease virus (NDV). *Rubulavirus* genus: parainfluenza virus type 4a (PIV4a); mumps virus (MuV); porcine rubulavirus (PoRV); Mapuera virus (MaV); simian virus 5 (SV5), now known as parainfluenza virus type 5 (PIV5); simian parainfluenza virus 41 (SV41); human parainfluenza virus type 2 (hPIV2). *Morbillivirus* genus: canine distemper virus (CDV); measles virus (MeV). Unclassified genus: Salem virus (SalV); Tupaia papamyxovirus (TPMV). This figure was adapted from (35).

1.2 Paramyxovirus Genome and Replication

Paramyxovirus viral genomes are of negative polarity running from 3' to 5' and range in size from 15 to 19kb in length. Paramyxoviruses contain 6 to 10 tandem genes with a typical gene order of N/NP–P/V–M–F–HN/H/G–L (Fig. 1-2). The nucleocapsid protein (N or NP) is produced from the N/NP gene. It is primarily responsible for encapsidation of the (-) sense RNA viral genome resulting in the formation of ribonucleoprotein complexes (RNPs); however, N protein can also non-specifically encapsidate (+) sense cellular RNA (83). The viral RNPs are typically characterized by a herringbone morphology, while cellular RNPs complexes are more heterogeneous in size and shape. The N protein also has the following additional roles: preventing recognition of viral RNA by the host immune system, protection of the viral genome from RNA degradation, incorporation of genomes during particle assembly, and serving as a platform for viral genome replication (83). The paramyxovirus P/V gene encodes the phosphoprotein (P), which is a critical component of the viral RNA-dependent RNA polymerase (RdRP). A second critical component of the RdRP is the large (L) protein encoded by the L gene. Although L protein contains all the catalytic activity of the polymerase, interaction with P protein is required for binding with viral RNPs (62). In addition to P protein, the P gene also produces V, W, and C proteins, which will be discussed in detail later in this chapter. The matrix (M) protein is produced from the M gene and is considered to be the main coordinator of virus particle assembly and release from the host plasma membrane. The F gene encodes the fusion (F) protein, which is a type I integral membrane glycoprotein responsible for fusion of the virus particles with the host plasma membrane. The second glycoprotein, hemagglutinin (H), hemagglutinin-neuraminidase (HN), or glyco- (G) proteins are type II integral membrane glycoproteins produced by the H, HN, or G genes, respectively. This group of proteins

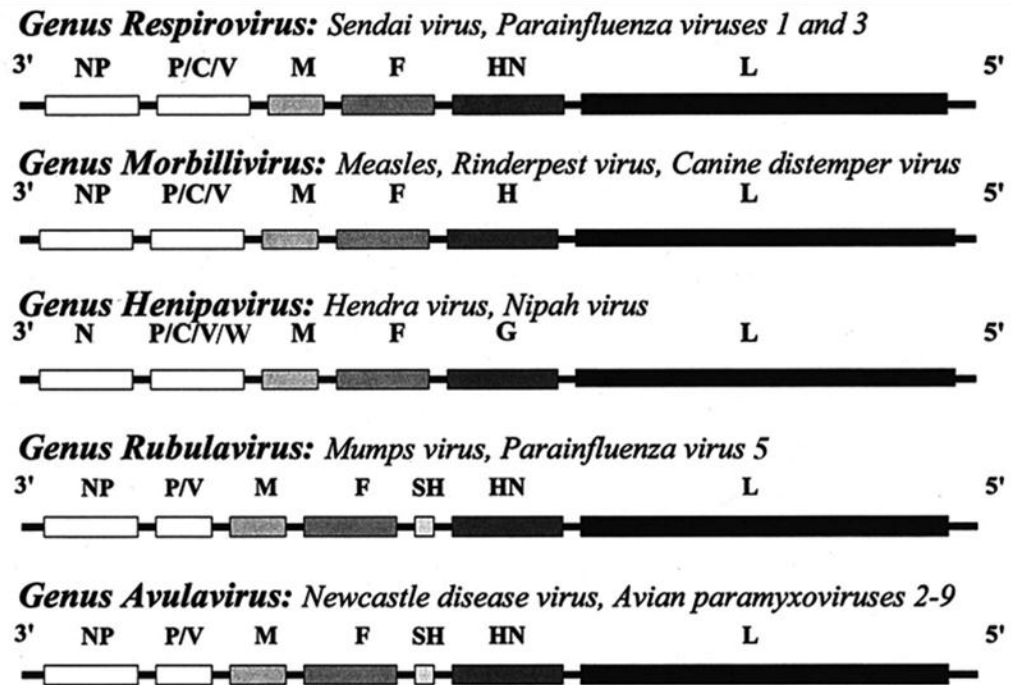
are responsible for recognition and binding to cellular receptors on the host cell surface (83). Paramyxoviruses that encode the HN glycoprotein utilize its neuraminidase activity to cleave sialic acid at a late step in the virus life cycle to prevent self-agglutination of viral particles on the cell surface in order to allow for their release.

Virus particle entry can only occur following processing of F0 into F1 and F2 products by cellular proteases. The cleavage of F0 in paramyxoviruses typically occurs at multibasic residues during trafficking through the *trans*-Golgi network by furin (Reviewed in 76). However, SeV, hPIV1, and an avirulent form of NDV only contain a single basic residue and require secreted endoproteases to cleave F0 following transport to the cell surface. Alternatively, the NiV F protein, unlike most paramyxoviruses, does not require any basic residues for cleavage (107). The NiV F0 cleavage also occurs following endocytosis of F0 from the cell surface, at which point NiV F0 is cleaved within endosomes by cathepsin L and trafficked back to the cell surface (31, 105, 112). Despite these different methods of paramyxovirus F0 processing, the F1 and F2 products are ultimately bound together by disulfide bonds and serve to penetrate host cellular membranes following virion attachment by the HN/H/G proteins. The HN protein of PIV5, MuV, SeV, and NDV attach to a host cell through binding with the common receptor, sialic acid (83). However, other paramyxoviruses utilize alternative receptors for virus entry such as SLAM or EphrinB2/B3 for MeV or *Henipaviruses*, respectively (110, 150). The binding of the viral attachment protein induces a conformational change in the F protein leading to fusion of virus-cell membranes and delivery of viral RNPs.

Following virus entry, the RdRP begins primary transcription of mRNAs from incoming viral RNPs. Paramyxovirus RNA synthesis occurs in the 3' to 5' direction and upon encountering cis-acting elements, located at intergenic regions, the RdRP may fail to reinitiate transcription of the next viral gene. This process, known as 'start-stop' transcription, results in production of a gradient of mRNAs with the highest levels being

produced from genes located closest to the 3' end of the genome. The mRNA produced is polyadenylated and capped by the viral L protein to prepare the mRNA for translation by the host translation machinery (83). When sufficient levels of viral proteins have accumulated, genome replication begins with the production of (+) sense antigenomes. These antigenomes are then used for production of (-) sense viral genomic RNA. Abundant accumulation of (-) sense genomic RNAs leads to secondary transcription of viral mRNA and ultimately packaging of viral RNPs into new virions for release.

A



B

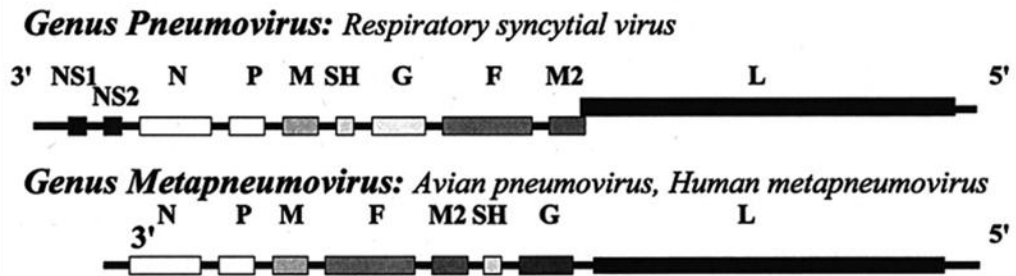


Fig. 1-2 Paramyxovirus genome organizations.

(A) Genomic structures of the Paramyxovirinae subfamily with represented viruses shown for each genus. (B) Genome structures of the Pneumovirinae subfamily with representative viruses shown for each genus. This figure was adapted from (34).

1.3 Paramyxovirus Assembly and Budding

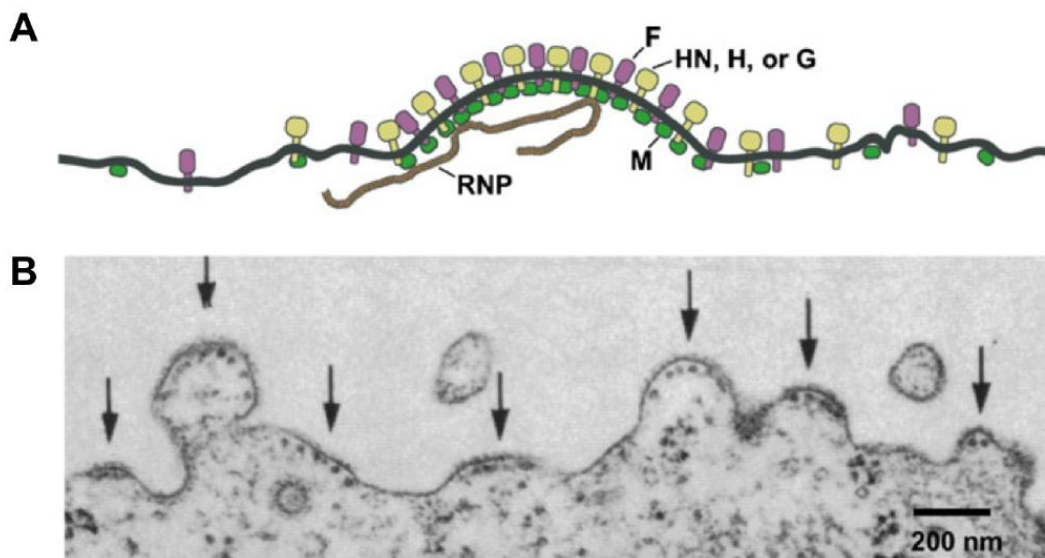
Paramyxovirus budding is the summation of a series of successful steps of the virus life cycle leading to the recruitment of viral proteins and the viral RNP to discrete sites on cellular membranes, depicted in Figure 1-3 (54). These components are assembled and pinched off from the cellular membrane following a fission event. The resulting virus particle is composed of a bilipid membrane derived from the host cell that is typically spherical in shape; however, virus particles can also be filamentous or pleomorphic. Virus particle size is highly variable with the majority of paramyxoviruses having particles ranging in size from 150 to 300 nm in diameter, although particle diameters exceeding 1 μm have been observed for *Henipaviruses* (47).

Virus particles are adorned with two glycoprotein spikes (F and either HN, H, or G proteins) along the surface that are responsible for the attachment and entry into a host cell. Within the viral particle is the viral RNP, which occupies the majority of the inner space of the particle. Also associated with the viral RNP is the viral phosphoprotein (P) and large (L) protein that are critical for initiation of viral replication. The matrix (M) protein is the coordinator of assembly for these viral components during particle formation (Fig. 1-4). The M protein lines the inner leaflet of the lipid membrane and acts as a bridge between the viral RNP and the surface glycoproteins. Assembly of these viral components occurs through M protein interactions with the viral RNPs and the cytoplasmic tails of the glycoproteins.

The critical role that M protein plays in the assembly and budding of paramyxoviruses was best described upon the advent of reverse genetics virus systems. This technology allowed for the production of M protein null recombinant viruses, which completely lack M protein. Recombinant MeV and Sendai virus (SeV) that lack M protein were completely defective in their ability to form particles (10, 70). Without a

functional M protein to mediate the release of particles, these viruses could only spread via extensive syncytia formation caused by an increase in the accumulation of active glycoproteins on the cell surface.

Virus fusion, or syncytia formation as seen with M protein null viruses, requires energy stored within the metastable conformation of the fusion (F) protein to overcome the energy required to fuse the two membranes. The reciprocal process of virus budding also requires energy to pinch off particles from the host-derived membrane. As a result, many enveloped viruses recruit host factors to assist in the final steps of virus particle production. One common cellular complex hijacked by enveloped viruses for budding is the endosomal sorting complex required for transport (ESCRT). The recruitment of host factors, like ESCRT, is critical for the efficient production of virions for many paramyxoviruses.



Harrison, et al., *Int. J. Biochem. Cell Biol.* 2010

Fig. 1-3 Production of virus-like particles.

(A) A depiction of assembly of viral proteins at the plasma membrane, with M protein forming protein-protein interactions with both the RNP and surface glycoproteins. **(B)** Thin section TEM of SeV budding from the plasma membrane of MDBK infected cells. Arrows represent individual virus budding sites. RNPs can also be visualized aligning just below the plasma membrane. This figure was adapted from (54).

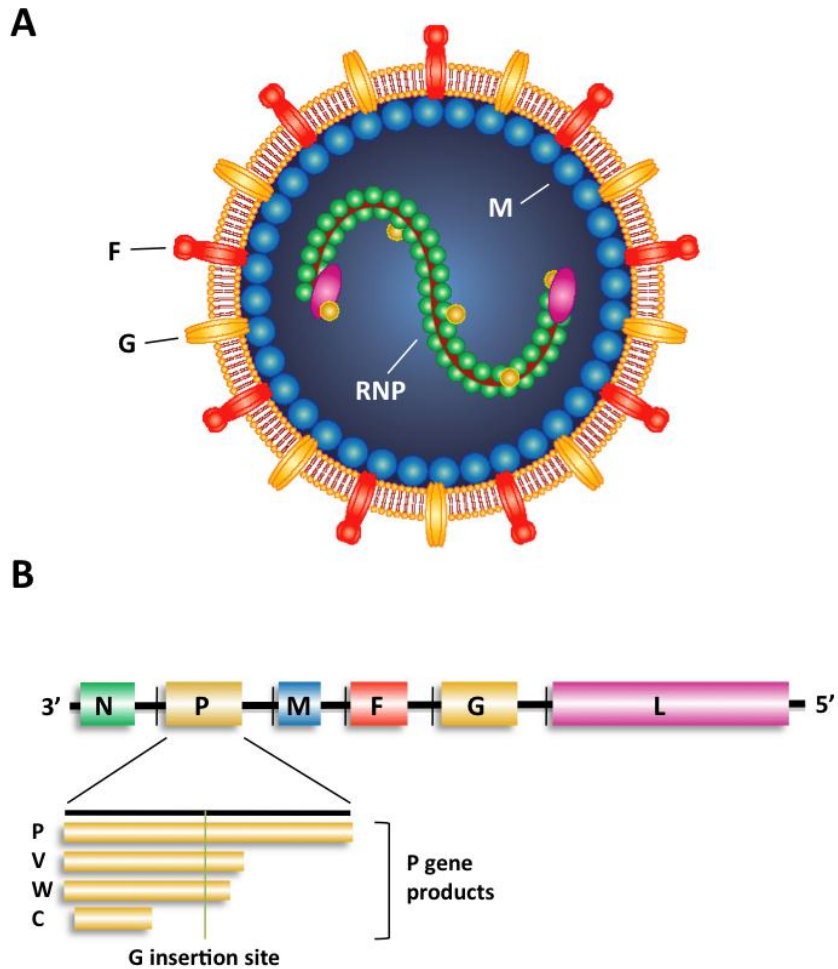


Fig. 1-4 *Henipavirus* particle and genome.

(A) A schematic representation of a *Henipavirus* particle. This enveloped virus is studded with the viral glycoproteins (F and G) at the surface of the particle. The matrix (M) protein is coating the inner leaflet of the lipid bilayer and is forming interactions with the cytoplasmic tails of the glycoproteins. The RNP consists of the viral genome encapsidated by the nucleoprotein (N) and also associated with RdRP consisting of large (L) and phospho- (P) proteins. Viral proteins are color coded with the genome below. **(B)** A detailed view of the P gene products. P, V, or W gene products share identical N-termini and only differ beyond the cis acting element. Up to eleven additional guanine nucleotides are added resulting in unique C-termini for each gene product. The C gene product is the result of translation initiation at an alternate start codon in a process known as leaky scanning.

1.4 Host Factors Required for Enveloped Virus Particle Budding

Numerous cellular factors have been identified to be important for enveloped virus assembly and budding; however, the cellular ESCRT machinery has been implicated in the budding of a broad range of viruses. ESCRT is comprised of four subunits: ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III each of which is composed of a smaller network of proteins. These four subcomplexes work together for the transport of cellular components along the endocytic pathway into multivesicular bodies (MVBs). Often this cellular cargo within MVBs is delivered to lysosomes for degradation. The ESCRT machinery was originally identified in *Saccharomyces cerevisiae* and was termed class E VPS (Vacuolar Protein Sorting) proteins (14, 128). Many human homologs of yeast class E VPS components have subsequently been identified as important host factors required for enveloped virus budding.

The amino acid motifs responsible for virus-ESCRT interactions were originally characterized in retroviruses. These motifs were termed late or L domains due to their involvement in the late stages of virus replication (Reviewed in 15). Of these motifs, three have been identified to be critical for the recruitment of specific ESCRT components: PTAP, PPXY, and YP(X)_nL. The polyprotein gag of human immunodeficiency virus type 1 (HIV-1) requires the PTAP motif to recruit the ESCRT-I subunit tumor susceptibility gene 101 (TSG101), for virus particle budding (44). Removal of the PTAP motif causes HIV-1 particles to remain attached to the cell surface by membrane stalks, resulting in reduced virus budding and infectivity (44). Another late domain motif YP(X)_nL, is required for interaction with the host protein AIP1/Alix. AIP1 is a component of the ESCRT machinery, which interacts with subunit in both ESCRT-I and ESCRT-III. The recruitment of AIP1 is critical for the budding of HIV-1, equine infectious anemia virus (EIAV), and SeV (73, 146). However, the ATPase VPS4 is the

linchpin to the entire ESCRT complex. Overexpression of a dominant negative form of VPS4 that lacks ATPase activity causes severe budding defects in enveloped viruses that require ESCRT. This is one of the most common methods used to determine the importance of ESCRT machinery for enveloped virus assembly and budding.

Despite lacking the well-characterized PTAP, PPXY, and YP(X)_nL late domain sequences, the budding of MuV and parainfluenza virus 5 (PIV5) still require the ESCRT machinery for particle release (87, 135). The recruitment of ESCRT by PIV5 M protein is mediated through a late domain motif FPIV, however the exact mechanism of ESCRT recruitment is still unknown (135). In addition to requiring the ESCRT machinery, PIV5 M protein also interacts with caveolin-1 (Cav-1) to facilitate the concentration of viral components at sites of virus assembly (126). The depletion of Cav-1 results in the inappropriate incorporation of cellular proteins and reduced particle infectivity (126). Interestingly, the PIV5 M protein binding site described for Cav-1 is also very close to a 14-3-3 binding site (126). The interaction between PIV5 M protein and 14-3-3 serves as a negative regulator of virus particle production (120, 126). This raises the possibility that Cav-1 and 14-3-3 compete for binding with PIV5 M protein, potentially providing an internal regulatory mechanism for PIV5 virus particle production.

Some enveloped viruses including, HRSV, influenza, NiV, and HeV, release virus particles independent of ESCRT machinery. HRSV and influenza virus both require the RAB11-FIP2/3 complex for virus assembly and budding. RAB11 is a small GTPase involved in endocytic trafficking that plays a critical role in late steps of cytokinesis, a process that resembles enveloped virus budding when in complex with FIP3 (168). Inhibition of the RAB11-FIP3 complex using siRNA knockdowns of either RAB11 or FIP3 leads to defects in the late stages of influenza virus budding (8). This defect resembles the accumulation of enveloped virus particles at the cell surface following disruption of the ESCRT machinery. Similar to influenza virus, HRSV also depends on RAB11

although its assembly and budding requires a different RAB11 interacting protein, FIP2 (154). The assembly and budding of the recently emerged *Henipaviruses*, NiV and HeV, also occurs independent of ESCRT machinery (118). Currently there are no known alternative host factors that contribute to *Henipavirus* assembly and budding. This highlights the need to identify novel virus-host interactions necessary for *Henipavirus* release.

1.5 Virus-like Particle Production

The process of virus release has been well-studied and many laboratories have taken a reductionist approach to characterize the requirements for virus particle release. For many retroviruses, expression of the gag polyprotein by itself is sufficient for virus-like particles (VLPs) production (Reviewed in 29). VLPs that are produced through overexpression of just a single protein, such as the retroviral gag protein, are morphologically similar to authentic virions but completely non-infectious. In addition to being non-infectious, VLPs can also be highly immunogenic. These properties of VLPs make them particularly important for the study of highly-pathogenic viruses and for vaccine development. The use of VLPs as a vaccine was demonstrated during the development of the VLP-based human papillomavirus (HPV) vaccine to help prevent cervical cancer (Reviewed in 93). In this case, HPV-like particles used for vaccination are mass-produced in yeast by expression of the major capsid protein, L1, which provided an economically viable method for vaccine production.

The diverse requirements for efficient production of VLPs have been described for many paramyxoviruses. In many cases, co-expression of multiple viral proteins can increase VLP budding efficiency. For PIV5, co-expression of M, NP and either glycoprotein, HN or F, are required for efficient VLP release (136). The closely related MuV, shares similar requirements for efficient VLP production (87). For many other

members of the paramyxovirus family such as NDV, MeV, and NiV, expression of M protein by itself leads to efficient VLP release (19, 113, 117, 123, 148). In some cases, the F protein can also release in vesicles completely independent of M protein, which is the case for SeV, MeV and NiV (117, 123, 148). For NiV, a third protein, G, can also form vesicles albeit much less efficiently than NiV F protein (117). Non-structural proteins such as SeV C protein have also been shown to enhance VLP formation (72, 131, 148). Taken together, paramyxoviruses demonstrate a wide variety of viral protein requirements to successfully escape from the cell.

1.6 Multifunctional Paramyxovirus P Gene

The P/V genes of paramyxoviruses encode multiple proteins via a polymerase stuttering mechanism during transcription and leaky ribosomal scanning. During transcription, up to 11 non-templated G residues can be inserted into P/V gene mRNA transcripts at a UC-rich region, leading to frame shifts (80, 91). This results in production of up to three proteins, P, V, and W, with identical amino termini but unique carboxyl termini created from each reading frame (57). Additionally, paramyxoviruses direct the production of a unique P gene product by taking advantage of leaky ribosomal scanning during translation. This process occurs because the primary translational start site of P protein is in poor Kozak consensus sequence, occasionally resulting in translation initiation at alternate sites. As a consequence, paramyxoviruses can produce between 1 and 4 viral C proteins from any of the P gene transcripts. This mechanism is utilized by a wide variety of paramyxoviruses including: MeV, SeV, hPIV1, hPIV3, NiV, and HeV. Both NiV and HeV produce four P gene products, P, V, W, and C proteins. Although the overall numbers of P, V, or W transcripts produced during the course of NiV infection are similar, their production is temporally regulated (80, 91). The

NiV P protein transcripts are produced first, likely to help to establish infection, while both V and W transcripts arise later during infection to suppress IFN-signaling (80).

Paramyxovirus P proteins are an essential component of the RdRP, playing a role in both viral mRNA transcription and genome replication. For viral mRNA transcription, the carboxyl region of P protein is sufficient for all aspects of viral mRNA transcription as demonstrated by work with the SeV P protein (25). The P protein carboxyl region is critical for linking L protein with the encapsidated viral RNA through direct interactions with L protein and the N:RNA complex (62). In contrast to viral mRNA transcription, genome replication requires the amino-terminus of paramyxovirus P protein. The amino-terminus of P protein functions to prevent unassembled N protein from non-specifically encapsidating cellular RNA. Since only encapsidated viral RNA can serve as a template for genome replication, prevention of non-specific cellular RNA encapsidation is critical to promote virus replication.

The V protein, expressed by many paramyxoviruses, is a multifunctional protein that plays roles in suppression of viral genome replication and in disruption of the innate immune response. Since V protein shares the amino-terminus with P protein it is thought to be able to compete with P protein for binding to soluble N protein. The binding of V and N proteins has been demonstrated for PIV5, SeV, and MeV V proteins (63, 125, 153). It is likely that this V-N protein interaction, unlike the P-N protein interaction, suppresses viral genome replication by preventing P protein from properly directing viral genome encapsidation. A second major function of V protein is to disrupt the innate immune response. Several paramyxovirus V proteins can interfere with the cell's ability to respond to IFN-signaling. Paramyxovirus V protein's disruption of IFN-signaling is typically accomplished through regulation of signal transducer and transcription activator (STAT) proteins by directing their degradation, sequestration of STAT proteins in high molecular weight complexes, or by preventing nuclear

translocation of activated STAT (Fig. 1-5). The ability to disrupt IFN-signaling by paramyxovirus V proteins can be critical for productive virus infection to occur; and in some cases it can determine the host range of the virus.

The most well characterized paramyxovirus C proteins are the four C proteins, C', C, Y1 and Y2, encoded by SeV. SeV C proteins were first suggested to have a role in counteracting the interferon (IFN) mediated innate immune response by targeting STAT1 for degradation or by preventing STAT1 phosphorylation (43). These data provided evidence of a dual mechanism for SeV C protein suppression of IFN-signaling. Interestingly, an N-terminal 23 amino acid extension encoded by only the C' and C forms of C protein was sufficient to bind and degrade STAT1 (42). Further investigation of this sequence identified it as a membrane targeting sequence critical for STAT1 degradation by SeV C' and C proteins (96). This finding shed light on why only SeV C' and C proteins were able to induce STAT1 degradation, while the SeV Y1 and Y2 proteins only prevented STAT1 phosphorylation through a secondary STAT1 binding site common to all forms of SeV C protein.

In addition to disruption of IFN-signaling, development of a recombinant null C protein SeV, 4C(-), led to the identification of a role for SeV C protein in virus particle production. Infections performed *in vitro* with the 4C(-) SeV identified a severe replication defect with viral titers several logs lower than wild type virus (81). Subsequent studies demonstrated that SeV C' or C protein can enhance Sendai VLP production by 2-3 fold while the Y1 and Y2 proteins cannot, thus implicating SeV C protein as a contributor to SeV assembly and budding (72, 148). This characteristic difference between the SeV C proteins was again linked to a difference in the membrane binding potential of the proteins. The membrane targeting sequence that is absent in the Y1 and Y2 proteins did not allow them to promote Sendai VLP production (72). In addition to binding membranes, SeV C protein also binds with the ESCRT component

AIP1 (131). The binding of SeV C protein with AIP1 and cellular membranes are required for SeV C protein to enhance VLP production (72). However, the requirement for AIP1 and ESCRT in SeV assembly and budding has subsequently been challenged. *In vitro* SeV infections, following depletion of AIP1 and/or disruption of ESCRT using dominant negative-VPS4A, found no defect in infectious virus particle production (49). Although there is no clear explanation for the observed differences, the discrepancy may be a result of the utilization of different pathways for Sendai VLP and infection based assays. Or, that during SeV infection other viral components not present in VLP based assays can compensate for the lack of ESCRT function.

Like SeV C protein, NiV C protein is multifunctional and has been implicated in inhibition of IFN-signaling and regulation of viral genome replication. Experiments performed *in vitro* have demonstrated that NiV C protein can inhibit IFN-signaling and allow an IFN-sensitive virus, VSV, to grow in tissue culture (116, 174). Although NiV C protein can inhibit IFN-signaling, this function is not critical during virus infection as other P gene products serve as redundant methods of inhibition (174). Another function attributed to NiV C protein is the regulation of viral genome replication. Overexpression of NiV C protein in a minigenome replication assay was able to suppress replication by 3-fold (141). Additionally, NiV C protein was also able to suppress MeV minigenome replication suggesting that NiV C protein may have an overlapping functionality between multiple paramyxovirus genera (141).

Additional insight into the function of NiV C protein during virus infection has been gained through the use of a recombinant virus, rNiV (C-), which lacks NiV C protein expression. Characterization of rNiV (C-) found that without C protein NiV exhibits slower rates of growth and lower maximum titers without impairing the virus's ability to suppress an IFN-signaling (20, 98, 174). Additionally, hamsters infected with rNiV (C-) exhibited dramatically reduced signs of disease (98, 174). Cytokine profile analysis of

rNiV (C-) infected tissue culture cells found elevated levels of inflammatory cytokine production as compared to wild type NiV (98). Together, these data suggest that NiV C protein is a critical determinant of NiV pathogenesis and may control a host ability to mount a chemokine-induced immune response against infection.

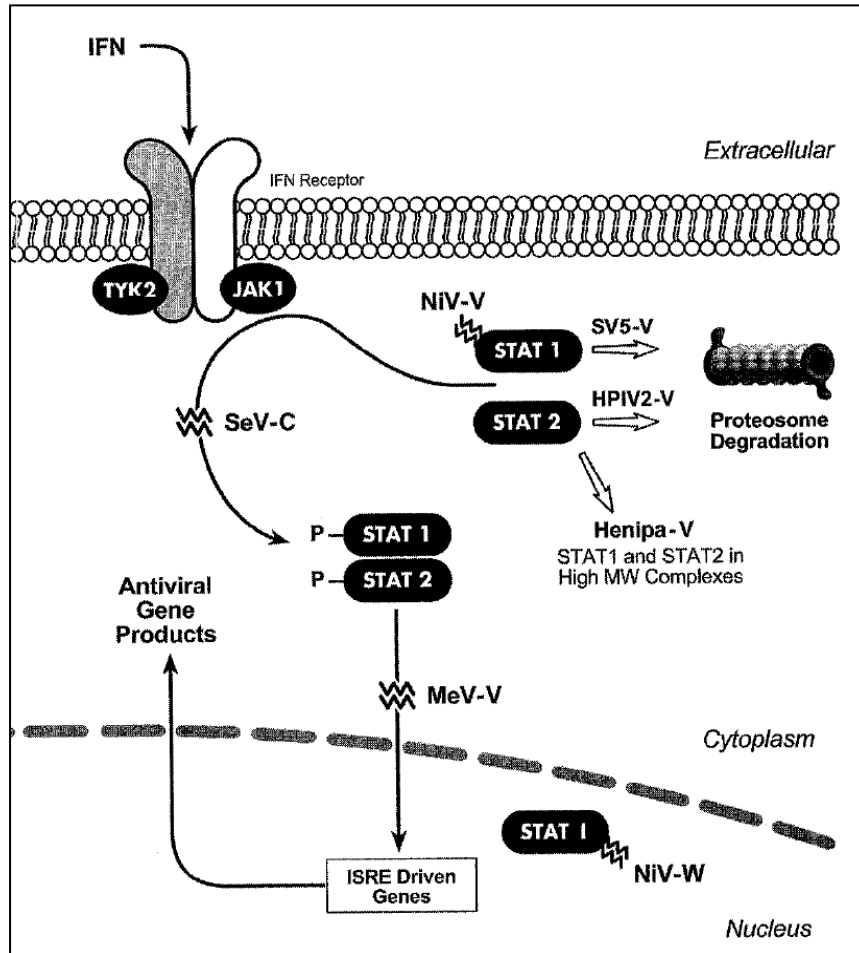


Fig. 1-5 IFN Induction, signaling and sites of inhibition

A schematic depicting various paramyxovirus strategies for inhibition of IFN induced STAT signaling by P gene products. Inhibition occurs through proteasome degradation of STATs (PIV5 and hPIV2 V proteins), sequestration of STATs in complexes (Henipavirus V and W proteins), preventing activation of STATs (SeV C protein), and preventing STATs nuclear translocation (MeV V protein). Figure modified from (83).

1.7 Ubiquitin-Proteasome and the N-end-rule Pathways

Ubiquitination is a type of post-translational modification where ubiquitin, an 8-kDa protein, is covalently attached to a lysine residue on a substrate protein. How a ubiquitin ligase attaches ubiquitin to a substrate protein has a large impact on the functional outcome. There are four types of ubiquitin attachment that have been well characterized: (i) monoubiquitination, (ii) multiple monoubiquitination, (iii) K63 polyubiquitination, and (iv) K48 polyubiquitination. Monoubiquitination or multiple monoubiquitination occurs when a single ubiquitin molecule is attached on one or more lysine residues. This process has been associated with protein trafficking, virus budding, and a variety of other cellular processes (55, 97). Polyubiquitination occurs when additional ubiquitin molecules are linked to lysine residues within a single ubiquitin molecule already attached to a substrate protein. This process leads to the formation of a chain of four or more ubiquitin molecules where the site of chain extension determines the substrate protein's fate. Chain extension occurring at lysine 63 within ubiquitin has been linked to cargo endocytosis and activation of protein kinases, while chain extension on lysine 48 leads proteasome-dependent protein degradation. Proteasome-dependent degradation of proteins results in their breakdown into small peptides and allows for the recycling of ubiquitin.

Ubiquitin-mediated protein degradation was first described in 1985 and was quickly associated with an N-terminal degradation sequence called the N-end-rule pathway (2, 21, 60). The primary N-terminal destabilizing sequences can be categorized into two types, basic 'type 1' residues or hydrophobic 'type 2' residues. These destabilizing sequences serve to recruit UBR box-containing E3 ubiquitin ligases that polyubiquitinate the recruiting protein (Fig. 1-5). Slight derivatives of the N-end-rule pathway have also been described in mammals, yeast, and bacteria. The N-end-rule

pathway is also used by Sindbis virus and HIV-1 to degrade the viral RNA polymerase and integrase proteins, respectively (27, 108). The degradation of viral proteins by the N-end-rule pathway may serve to avoid adverse activity on their hosts.

The ubiquitination of viral gag or M proteins can also be critical for proper virus particle assembly and budding. However, ubiquitination for the purpose of virus particle production requires the viral proteins be monoubiquitinated. This requirement can be observed through depletion of free pools of ubiquitin by treating cells with proteasome inhibitors. In many cases, treatment with proteasome inhibitors reduces viral particle budding and the monoubiquitination of both retroviral gag proteins and the M proteins negative strand RNA viruses. For example, proteasome inhibitor treatment causes nuclear retention of NiV M protein, which leads to defects in virus budding and the membrane association M protein (162). This observation was linked to the ubiquitination of a single conserved lysine residue within a nuclear localization signal encoded by NiV M protein (162). Another approach to investigating the importance of monoubiquitination in assembly and budding is the cumulative removal of lysine residues. The removal of several lysine residues that were identified as sites of monoubiquitination in PIV5 M protein, lead to defects in virus budding, infectivity, and the cellular localization of PIV5 M protein (55). Although the importance of viral protein monoubiquitination is not completely understood, it has been proposed to be required for the recruitment of ESCRT components required for the budding of many enveloped viruses.

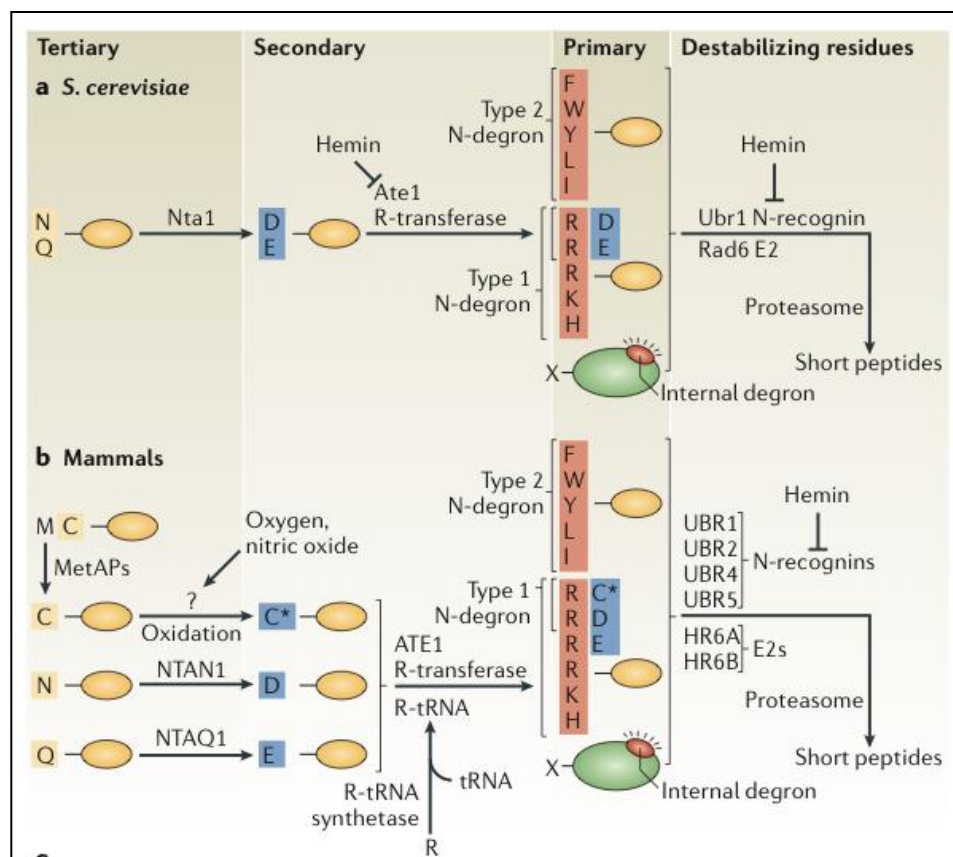


Fig. 1-6 N-end-rule pathway.

(A) A schematic of the N-end rule pathway in *Saccharomyces cerevisiae*. The tertiary destabilizing amino acids can become deamidated by the N-terminal amidase (Nta1) leading to formation of the secondary destabilizing amino acids. The Secondary destabilizing amino acids Asp and Glu are arginylated by R-transferase forming primary destabilizing residue. Together the type 1, type 2, and internal degron sequences are recognized by the E3 Ubr1/E2 Rad6 ubiquitin ligase complex leading to polyubiquitination and protein degradation. **(B)** In mammals, the process is similar except Asn and Glu are deamidated by NTAN1 and NTAQ into Asp and Glu, respectively. Additionally, Cys can be oxidized leading to arginylation along with Asp and Glu to primary destabilizing residues. The E2-E3 complex consists of UBR1, UBR2, UBR4, and UBR5 (E3s) with HR6A and HR6B (E2s). For both pathways Hemin can inhibit arginylation by R-transferases. Figure adapted from (144).

1.8 Identification of Protein-Protein Interactions

Since 1989, when the Fields lab first pioneered yeast two-hybrid screening, the system has proven to be an invaluable tool for the identification of protein-protein interactions across numerous biological systems (36). Yeast two-hybrid systems represent a genomics-based approach to identification of novel protein interactions. This approach exploits the knowledge that many transcription factors can be separated into independently functioning domains for DNA-binding and transcriptional activation. When these domains are individually fused to proteins of interest and an interaction occurs within yeast, the domains are brought together resulting in reconstitution of the transcription factor and subsequent activation of reporter genes. Yeast two-hybrid systems have been used to identify novel protein-protein interactions that are critical for virus particle production in a number of enveloped viruses. The ESCRT-I subunit, TSG101, was identified as a binding partner for HIV-1 Gag protein as a result of yeast two-hybrid screens (157). The subsequent characterization of the ESCRT complex has led to a greater understanding of virus budding not only in retroviruses, but also in other enveloped viruses such as Ebola virus, vesicular stomatitis virus (VSV), herpes simplex virus (HSV), and the avian coronavirus, infectious bronchitis virus (IBV) (24, 56, 152, 161).

In addition to yeast 2-hybrid screening, proteomics based assays such as co-affinity purification, have been used extensively to investigate protein-protein interactions between known proteins. However, this technology can also extend to screening for novel protein-protein interactions. Co-affinity purifications typically utilize a GST, Biotin or His6 affinity tag fused to a bait protein of interest and untagged prey proteins. Following purification of the bait protein, co-purified candidate prey proteins are identified using mass spectrometry. However, the candidate binding proteins identified do not

necessarily form a direct interaction with the bait protein. This is unlike candidates isolated from yeast 2-hybrid screening, which selects for direct protein-protein interactions. As a result, large protein complexes are typically co-purified with the bait protein of interest and the detection of direct protein-protein interactions can be more challenging. However, the purification of large protein complexes can yield valuable insight into protein networks and pathways that are relevant to the bait protein of interest.

Co-affinity purification has been used to identify virus-host protein interactions. One such interaction was identified between Ebola VP40 protein and a member of the COPII vesicular transport system, Sec24C (171). This interaction proved to be critical for the cellular trafficking of Ebola VP40 protein and provided a novel target for potential antiviral intervention. One other example of utilization of this technique was in the identification of numerous host cell factors potentially important for influenza RNP complex function (101). Co-affinity purification strategies have proven to be useful tools for identification of protein-protein interactions important for viral protein function.

1.9 Preview

Several paramyxoviruses require cooperation between host and viral proteins to efficiently produce VLPs (16, 87, 89, 113, 136, 148). The aims of this study were to characterize viral and host factors that are important for *Henipaviruses*. We found that the NiV C protein did not play a role in Nipah VLP production despite being able to release into the media. Additional characterization of NiV C protein revealed that the release into the media was dependent on binding with cellular membranes via an N-terminal region containing several hydrophobic and basic residues. This N-terminal region was not only required for membrane-binding but it alone was sufficient to target a heterologous protein to membranes. We also observed that NiV C protein was highly

unstable in tissue culture and this instability was dependent on the ubiquitin-proteasome pathway. Characterization of NiV C protein ubiquitination found that the ubiquitinated species could be eliminated through cumulative mutation of all 14 encoded lysine residues resulting in partial protein stability. Alternatively, NiV C protein could be stabilized through mutation of the N-terminal basic region that plays a role in membrane-binding. Interestingly, this mutant was still heavily ubiquitinated suggesting that mono- and poly- ubiquitination may be occurring on NiV C protein. Overall, we have defined several molecular characteristics of NiV C protein, which may play an important role in viral replication and pathogenesis.

In an effort to identify host factors important for *Henipavirus* particle production both genomic (Y2H) and proteomic (co-affinity purification) approaches were employed to identify novel *Henipavirus* M-interacting proteins. In the process of identifying numerous *Henipavirus* M-interacting proteins a new method was developed which greatly reduced the non-specific co-purification of RNA-binding host proteins and identified new candidate *Henipavirus* M-interacting proteins. Of the host proteins identified, the minimal region required for NiV M-binding was defined for AP3B1, ARL6IP4, and KBTBD7 (In preparation Sun, et al., unpublished). A comprehensive assessment of the NiV M-interacting host proteins found that many host proteins contained similar serine-rich acidic regions required for binding. Overexpression of the serine-rich acidic fragments inhibited Nipah VLP production. This finding, combined with siRNA knockdown experiments, demonstrated the importance of *Henipavirus* M protein interactions with serine-rich acidic host proteins (In preparation Sun, et al., unpublished).

Chapter 2

Materials and Methods

Plasmids

NiV cDNAs M, N, F and G were cloned into the eukaryotic expression vector pCAGGS. Strep (II) and 6xHis (WSHPQFEKHHHHHH), Flag (DYKDDDDK), and Myc (EQKLISEEDL) tags were appended to the N-terminus of both pCAGGS-NiV and pCAGGS-HeV M for various applications. The pCAGGS-NiV C was cloned from a NiV P gene cDNA and a Flag tag was appended to both the N- and C-termini. All site-directed mutants of NiV C protein were made by PCR mutagenesis and their sequence identities were confirmed by sequencing the entire NiV C gene. The HA-Ub a kind gift from Cecile Pickart, and pRK5-HA-Ubiquitin-KO (HA-UB KO) (Addgene plasmid 17603) have been described before (55). All host protein cDNAs were purchased from openbiosystems (Thermo Scientific, Lafayette, CO) and cloned into pCAGGS-Myc or pCAGGS-Flag using gene specific primers, in frame with an N-terminal epitope tag for protein detection. Plasmids pYESTrp-Jun, pHybLex/Zeo-Fos2, pHybLex/Zeo-Lamin, and pHybLex/Zeo were obtained from Invitrogen (Carlsbad, CA) and used as controls or to construct NiV and HeV M bait plasmids based on manufactures instructions. The library used for yeast 2-hybrid screening was purchased from Origene Technologies (Rockville, MD). The library was derived from the WI-38 (human lung fibroblast) cell line that contains 5.7×10^6 individual clones in the pJG4-5 yeast vector with an insert size range of 0.4-5.0 kbp.

Antibodies and affinity resin

To generate antibodies against NiV M and N proteins, the full-length cDNAs were cloned into pRSETB (Invitrogen, Carlsbad, CA) and the proteins were expressed in *Escherichia coli* by autoinduction (147). The recombinant His-tagged proteins were

purified using metal affinity chromatography and sent to Harlan Bioproducts for Science (Indianapolis, IN) for polyclonal rabbit antibody production. Polyclonal NiV F and monoclonal NiV G antibodies were kind gifts of Christopher Broder (Uniformed Services University of the Health Sciences, Bethesda, MD). The FLAG[®] magnetic affinity resin was purchased from Sigma-Aldrich (St. Louis, MO). The polyclonal antibody specific to enhanced green fluorescent protein (EGFP) was purchased from Clontech (Mountain View, CA). The monoclonal VU-1 antibody that detects ubiquitin was purchased from LifeSensors (Malvern, PA). The monoclonal antibody 9E10 that detects the Myc epitope tag was purchase from Sigma-Aldrich (St. Louis, MO). The polyclonal antibody specific to the LexA DNA-binding domain was purchased from LPBio.

Measurements of VLP production

To generate Nipah VLPs, 293T cells in 6-cm dishes grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum were transfected with plasmid DNAs by using Lipofectamine-Plus reagents (Invitrogen, Carlsbad, CA). The plasmid amounts per dish are as follows unless otherwise indicated: pCAGGS-NiV M, 0.5 µg; pCAGGS-HeV M, 0.5 µg; pCAGGS-PIV5 M, 0.5 µg; pCAGGS-NiV N, 70 ng; pCAGGS-NiV F, 0.75 µg; pCAGGS-NiV G, 0.9 µg; pCAGGS-NiV C, 0.5 µg; Host protein in pCAGGS 1.5 µg. At 24h. post transfection (p.t.) culture media was replaced with DMEM containing 1/10 the normal amounts of methionine and cysteine and 40 µCi of [³⁵S]Promix/ml. After an additional 18h. cell culture media was collected for VLP production analysis.

To analyze VLP production the culture media were centrifuged at 7.5k x g for 2 min to remove cellular debris. Media was then layered onto a 20% sucrose cushion (4ml NTE [10 mM Tris, pH 7.4; 1 mM EDTA; 100 mM NaCl]) and centrifuged at 110k x g for 1.5h. Pellets were resuspended in 0.9 ml of NTE and mixed with 2.4ml of 80% sucrose

in NTE. Floatation gradient was prepared by layering 3.6 ml of 50% sucrose and 0.6 ml of 10% sucrose in NTE to the top of the samples before centrifugation at 110k x g for 3h. Following centrifugation, 3.8 ml of the sucrose gradient was collected from the top, mixed with 10ml NTE and pelleted at 140k x g for 1.5h. Purified VLP pellets were resuspended in 2x protein lysis buffer (PLB) (1M Tris, pH 6.8; 15% dithiothreitol; 10% sodium dodecyl sulfate [SDS]; 37% Glycerol) and loaded on either 10% or 15% SDS-PAGE gels.

Cell lysates were prepared and immunoprecipitated as previously described (136). Briefly, harvested cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris, pH 7.4; 1% deoxycholate; 1% Triton X-100; 0.1% SDS) containing 150 mM NaCl, 100 mM iodoacetamide, and 1 mM phenylmethanesulfonyl fluoride [PMSF] and clarified before being immunoprecipitated with the appropriate antibodies against viral and/or cellular proteins. Protein detection and quantification was performed using a Fuji FLA-7000 laser scanner (FujiFILM Medical Systems, Stamford, CT). The budding efficiency was calculated as the specific counts of M protein in the media divided by M protein in the corresponding cell lysates and all samples were normalized relative to controls.

Transmission electron microscopy of Nipah VLPs

To produce Nipah VLPs for electron microscopy, 293T cells in 5x10-cm dishes were transfected with NiV M, N, and G plasmids to produce Nipah viral proteins. At 48h. p.t., the culture media was collected and Nipah VLPs were purified on sucrose gradients as described above. Nipah VLPs were resuspended in 100µl NTE and passaged 10 times through a 26-gauge needle. Ten µl of the Nipah VLP preparation was adsorbed onto carbon-coated Parldion copper grids and stained with 1% uranyl acetate. VLPs were examined using a Jeol JEM 1200 EX II electron microscope operated at 80kV.

Sedimentation gradient analysis of Nipah VLP density

The production of VLPs for density gradient analysis is the same as detailed above for electron microscopy analysis. The purified Nipah VLPs were added to the top of a continuous density gradient constructed by overlaying 650 μl of OptiPrep™ [iodixanol] in increasing concentrations (6%, 12%, 18%, 24%, 30%, 36%, 42%) that was allowed to sit for 5h. at 20°C. Sedimentation of Nipah VLPs through the gradient was accomplished by centrifugation of samples at 150k x g for 16h. Following centrifugation, 16 x 300 μl fractions were collected from the top of the gradient and weighed to determine the density of each fraction. From each fraction 50 μl was removed and mixed with PLB and resolved on 10% SDS-PAGE gels. Detection and quantification was performed as described above.

Co-immunoprecipitation binding experiments

To confirm protein interactions with NiV M protein, 6-cm dishes of 293T cells were transfected with 0.5 $\mu\text{g}/\text{dish}$ of NiV M plasmid with or without a Flag tag appended to the N-terminus of the protein and 1.5 $\mu\text{g}/\text{dish}$ of the specified virus or host protein plasmids. For radiolabeled experiments, cells were pulse labeled by starving cells DMEM lacking methionine and cysteine for 20min before 40 μCi of [³⁵S]Promix/ml was added to the media. Following a 3h. labeling period, cells were harvested and lysed in 1 ml of Co-IP buffer (20 mM Tris, pH 7.6; 100 mM NaCl; 1 mM EDTA; 0.5% nonyl phenoxyethoxyethanol [NP-40]; 1 mM phenylmethanesulfonyl fluoride [PMSF]) on ice for 10 min. Cell lysates were clarified then precleared with 20 μl of sepharose beads for 30min before a portion was removed to measure total protein expression while the remaining sample was used for co-immunoprecipitation. Lysates were incubated with antibodies for 3h. then antibody complexes were allowed to absorb into Protein A coated sepharose beads for 30min. Immunoprecipitated samples were washed three times with

Co-IP buffer to eliminate unbound protein and resuspended in PLB. Samples were boiled for 5 min before being loaded on 10% or 15% SDS-PAGE gels to be processed by autoradiography or immunoblotting if cells were left unlabeled.

Membrane fractionation

To identify the membrane-binding potential of NiV C protein, 10cm dishes of 293T cells were transfected with 2 µg/dish of NiV C.FI (or derivatives) plasmid DNA. 24h. p.t., the cells were starved and pulse labeled with 40 µCi of [³⁵S]Promix/ml. Following labeling cells were harvested and resuspended in 750 µl of a hypotonic Dounce buffer (25 mM NaCl; 50 mM Na₂HPO₄, pH 7.4). Cells were incubated on ice for 30min. and lysed using a dounce homogenizer. Dounced lysate was spun at low speed, 4,500 x g for 10min, to clarify lysate. PMSF was then added to the clarified lysate and 10% was collected and saved as the input control. Lysates were then combined with 1.5ml of 80% sucrose in NTE for floatation. Additional layers of 2ml of 50% sucrose and 0.6ml of 10% sucrose were overlaid to the top of the sample. Samples were then spun at 150k x g for 16h. at 4°C. in a swinging bucket rotor. Seven equal fractions were collected from the top of the gradient (0.65ml each) and an equal amount of RIPA buffer was added for analysis of proteins by immunoprecipitation as described above for VLP analysis. Images were generated using a Fuji FLA-7000 laser scanner (FujiFILM Medical Systems, Stamford, CT).

Immunofluorescence microscopy

Coverslips in 12-well plates were seeded with Vero cells (50-60% confluent) and transfected with 0.5 µg/well of NiV C-EGFP plasmids. 24h. p.t., the cells were fixed with 4% paraformaldehyde in PBS for 10 min. Cells were permeabilized by incubating with 0.1% saponin, 3% bovine serum albumin in PBS (Phosphate Buffered Saline reagents, pH 7.4) for 30 min. at room temperature. Cells were washed three times with PBS and

nuclei were stained with 4',6-diamidino-2phenylindole (DAPI). Cells were visualized with a Zeiss Axio Imager.M1 fluorescence microscope (Zeiss, Inc., Thornwood, NY). Images were captured using a Hamamatsu C10600-10B camera (Hamamatsu, Inc., Bridgewater, NJ).

Pulse-chase analysis of protein stability

293T cells in 6cm dishes were transfected with 1 µg/dish of NiV C.FI (or derivatives) plasmid. 24h. p.t. cells were pulse labeled with 40 µCi of [³⁵S]Promix/ml for 15 min before media was exchanged for cold chase media (DMEM, 10% fetal calf serum, with or without 10 µM N-(benzyloxycarbonyl)leucinylleucinylleucinal [MG-132]) . Cells were harvested at chase periods of 0, 30, 60, or 60 min with MG-132. Cells were lysed in RIPA buffer and immunoprecipitated as described above. Images were generated using a Fuji FLA-7000 laser scanner (FujiFILM Medical Systems, Stamford, CT). The protein stability was calculated as the specific counts of C protein at each time point divided by C protein at the 0 min time point.

Ubiquitination experiments

293T cells in 10cm dishes of were transfected with 1 µg/dish of NiV C.FI (or derivatives) plasmid, and 2µg/dish pMT123 plasmid encoding HA-Ub, or pRK5 plasmid encoding HA-UbKO as indicated. Where indicated cells were treated with either 20µM MG-132 or DMSO as a control for 3h. prior to harvesting otherwise 24h. p.t., the cells were harvested and lysed in RIPA buffer containing 150mM NaCl. The lysates were clarified and immunoprecipitated with FLAG[®] magnetic affinity resin for 3 hours. Following immunoprecipitation, resin was washed two times with RIPA buffer containing 0.3mM NaCl; two times with RIPA buffer containing 0.15mM NaCl; and once with 50mM Tris (pH 7.4), 0.25mM EDTA, and 150mM NaCl. Following washing, the samples were resuspended in PLB. Half of each sample was loaded on 15% SDS polyacrylamide gels

for immunoblotting with VU-1 α -ubiquitin antibody or whole protein staining with either Sypro Orange or Lucy 506 stain purchased from Sigma-Aldrich (St. Louis, MO).

Ubiquitination site prediction

The prediction of potential ubiquitination sites within NiV C protein was performed with BDM-Pub (<http://bdmpub.biocuckoo.org>). The amino acid sequence of NiV C protein (GenBank accession number: AAK29086) used for prediction was used for analysis by BDM-Pub. Predictive scores of greater than 0.8 were considered to be potential sites of ubiquitin attachment.

Yeast 2-hybrid screening of *Henipavirus* M protein

Yeast 2-hybrid screening protocol was adapted from previous work, mating protocols contained in Two-Hybrid Systems: Methods and Protocols and methods described in Hybrid Hunter™ (Invitrogen) (68, 142). The yeast strain EGY48 (MAT α), which contains a LexA-driven leucine reporter gene, was transformed with a premade cDNA library derived from human lung fibroblast cells and stored at -80°C in 25 mM Tris, pH 7.6, 65% glycerol, 0.1M MgSO₄ for later use. Frozen aliquots were thawed and tittered using serial dilutions to determine the yeast CFU for later yeast mating. The cDNA library provided by the vendor was contained in the pJG4-5 vector in which prey cDNAs are fused with the B42 transcription activation domain. An additional yeast strain L40 (MAT α), which contains LexA-driven histidine, uracil and beta-galactosidase reporter genes, was transformed with either of three bait plasmids: pHybLexZeo-NiV M, pHybLexZeoH-NiV M, or pHybLexZeo-HeV M, their selection was maintained with Zeocin.

For yeast 2-hybrid screening, L40 harboring each bait plasmid was first grown for 18-20 hours in YPAD/Zeo100 (1% yeast extract; 2% Peptone; 2% dextrose; 0.2g/L adenine; 100 μ g/ml Zeocin) at 30°C to reach a final density of 2-4 x 10⁷ cells/ml. Freshly

grown bait containing L40 yeast were mixed at a 2.5:1 (MAT α :MAT α) ratio with thawed aliquots of pretransformed EGY48 containing library plasmids. The yeast mixture was diluted to $\sim 10^8$ cell/ml and was resuspended in YCM (1% yeast extract; 1% peptone; 2% dextrose; pH 3.5) media. Yeast mating was allowed to occur and incubated at 30°C for 2h before being grown on an YCM pH 4.5 agar plate for 12h at 30°C. Following mating, diploid yeast was collected and library protein expression was induced in 100 ml of induction dropout media YC –WHULK/Zeo100 (0.12% yeast nitrogen base; 0.5% ammonium sulfate; 1% succinic acid; 2% Galactose; 1% Raffinose; 0.01% Adenine, Uracil, Arg, Cys, Leu, Thr, Trp; 0.005% Asp, His, Ile, Met, Phe, Pro, Ser, Tyr, Val; 100 μ g/ml Zeocin) for 6h. at 20°C. Following induction, protein-protein interactions were selected for on 150 mm YC –WHULK/Zeo 100 selection plates after 3-5 days of growth at 30°C. Growing colonies were selected and isolated on additional yeast selection plates.

Secondary screening was performed using beta-galactosidase colony filter assay. Briefly, small portions of isolated candidates were resuspended in water and spotted on nitrocellulose membranes. The spotted membranes were freeze-thawed 3X at -80°C to before being incubated with Z buffer (60mM Na₂HPO₄; 40mM NaH₂PO₄; 10mM KCl; 1mM MgSO₄; pH 7.0) containing 1mg/ml of X-gal (5-bromo-4-chloro-indolyl- β -D-galactopyranoside) for 2h at 30°C. Candidates positive for the beta-galactosidase colony filter assay were grown in selection media and the plasmid DNA was isolated and subjected to PCR to amplify library inserts. PCR products were sequenced at the Genomic Core Facility (University Park, PA) and identified using the GenBank database.

***Henipavirus M* protein affinity purification following RNase A treatment**

Co-affinity purification of *Henipavirus M*-interacting proteins was carried out using ÄKTAprime plus system (GE Healthcare Amersham Biosciences AB, Uppsala, Sweden).

293T cells in 10-cm dishes were transfected with 3 µg/dish of SH-HeV M, or control plasmids. 24h. p.t. cell lysates were harvested and lysed in buffer A (0.5% NP-40; 100 mM Trizma base, pH 8.0; 150mM NaCl; 1 mM EDTA) and 1 mM PMSF for 10 min on ice. Following lysis, samples were clarified by centrifugation and treated with 200 µg/ml of RNase A for 30 min. at 20°C. RNase A treated samples were clarified a second time by centrifugation then injected into a superloop on the ÄKTAprime plus system fitted with a 1 ml StrepTrap-HP column. Purification was performed by using the preprogrammed method for Affinity Purification with any HiTrap using buffer A to wash and buffer B (100 mM Trizma base, pH 8.0; 150mM NaCl; 1 mM EDTA; 2.5 mM Desthiobiotin) to elute. The eluted protein fractions were concentrated to 50 µl with U-Tube Concentrators, 500-3 (Novagen) and samples were mixed with PLB and resolved on a 10% SDS-PAGE gel for Coomassie Brilliant Blue (American Bioanalytical, Natick, MA) staining. Eight bands visible by Coomassie Brilliant Blue staining were excised (~210; ~130; ~120; ~114; ~112; ~110; ~70; ~35 kDa) and sent to the Taplin Mass Spectrometry Facility (Harvard Medical School, Boston, MA) for protein identification.

Chapter 3

Molecular Characterization of NiV C Protein

3.1 Introduction

Enveloped viruses commonly require cooperation between multiple viral proteins in order to efficiently produce virus-like particles (VLPs) that resemble authentic virions (16, 87, 89, 136, 148). However, Nipah virus (NiV), a zoonotic paramyxovirus that naturally infects bats and can cause fatal vasculitis in humans, only requires the matrix (M) protein for efficient VLP production (18, 19, 117). Here, we have set out to confirm these previous findings and expand on the requirements for Nipah VLP production by assessing the relative contribution of a nonstructural protein, NiV C. The involvement of C protein in virus particle production has been shown for another paramyxovirus, Sendai virus (SeV). SeV C protein can enhance Sendai VLP production to levels similar to SeV infection when co-expressed with SeV M, N, F, and HN proteins (72, 131, 148). SeV C protein's role in VLP production was later found to require the host factor AIP1/Alix (131). However, the involvement of AIP1 in SeV assembly has subsequently been disputed (49).

In addition to enhancing Sendai VLP production, SeV C protein binds with cellular membranes. The membrane-binding of SeV C protein requires an N-terminal membrane targeting sequence, which consists of basic and hydrophobic residues (96). The membrane targeting sequence consists of the first 23 amino acids of SeV C protein, and this sequence alone is sufficient for SeV C protein membrane-binding. The fusion of the SeV C protein membrane targeting sequence to enhanced green fluorescence (EGFP) redirected EGFP to cellular membranes (96). A sequence comparison between the N-termini of SeV C and NiV C proteins has revealed that NiV C protein also contains

similar N-terminal characteristics that may also allow NiV C protein to bind with cellular membranes.

Paramyxovirus C proteins are multifunctional proteins with roles in suppression of IFN-signaling, regulation of viral genome replication, and viral pathogenesis. The measles virus (MeV), human parainfluenza virus type 1 (hPIV1), and SeV C proteins can all disrupt the host's IFN-signaling pathway. All three viral C proteins directly bind with STAT1; however, SeV C protein induces STAT1 degradation while hPIV1 and MeV C proteins prevent nuclear translocation during virus infection (41-43, 137, 172, 173). In addition to suppression of the IFN response, MeV and SeV C proteins can regulate viral genome synthesis (4, 41, 43, 51, 71, 127). Similarly, NiV C protein can disrupt cellular IFN-signaling and regulate viral genome replication (54, 68). NiV C protein is also involved in viral pathogenesis independent of regulating the host immune response (116, 141, 174). This may be directly linked to new findings that NiV C protein inhibits the expression of proinflammatory cytokines in response to viral infection (98). Together, these observations provide insight into the function of NiV C protein during viral infection. However, the molecular characteristics that likely play a critical role in these functions are still poorly defined.

3.2 Results

The contribution of NiV glycoproteins to Nipah VLP production

To demonstrate how VLP production can vary among paramyxoviruses, NiV, Hendra virus (HeV) and parainfluenza virus type 5 (PIV5) M proteins were expressed in HEK293T cells and metabolically labeled with ³⁵S. Following labeling, VLPs were purified on sucrose floatation gradients and cell lysates were immunoprecipitated with M protein antibodies. Comparison of the VLPs produced through expression of viral M proteins by themselves, found that only the *Henipavirus* M proteins and not PIV5 M

protein were sufficient for efficient VLP release into the media (Fig. 3-1A). This finding demonstrates the clear differences in the requirements for VLP release that can exist among paramyxoviruses and is consistent with our previous observations (87, 136).

Other groups have reported that only expression of NiV M protein by itself was required for efficient Nipah VLP production (19, 117). However, due to differences in our protocol used to produce Nipah VLPs, as compared to others, we examined the relative efficiency of VLP production with varying combinations of NiV M, nucleocapsid (N), glyco- (G) or fusion (F) proteins (Fig. 3-1B). We found that expression of the NiV G protein by itself allowed for limited release of vesicles containing NiV G protein into the media. Similar to NiV G protein, the NiV F protein released vesicles into the culture media but to a much greater level as compared to NiV G protein. Additionally, both NiV F protein's cleaved and uncleaved form, F₁ and F₀ respectively, were detected in the culture media. The only viral protein completely unable to release into the media was the NiV N protein. Although several viral proteins can release into the media by themselves when they were co-expressed in combination, there were no significant differences in Nipah VLP production as compared to NiV M protein by itself (Fig. 3-1B,C). Nipah VLP production efficiency was measured based on the relative amounts of NiV M protein in the media for each of the viral protein combinations. These results are consistent with the previous reports that NiV M protein alone is the major driving force of budding and that other viral proteins do not contribute to Nipah VLP release (20, 117).

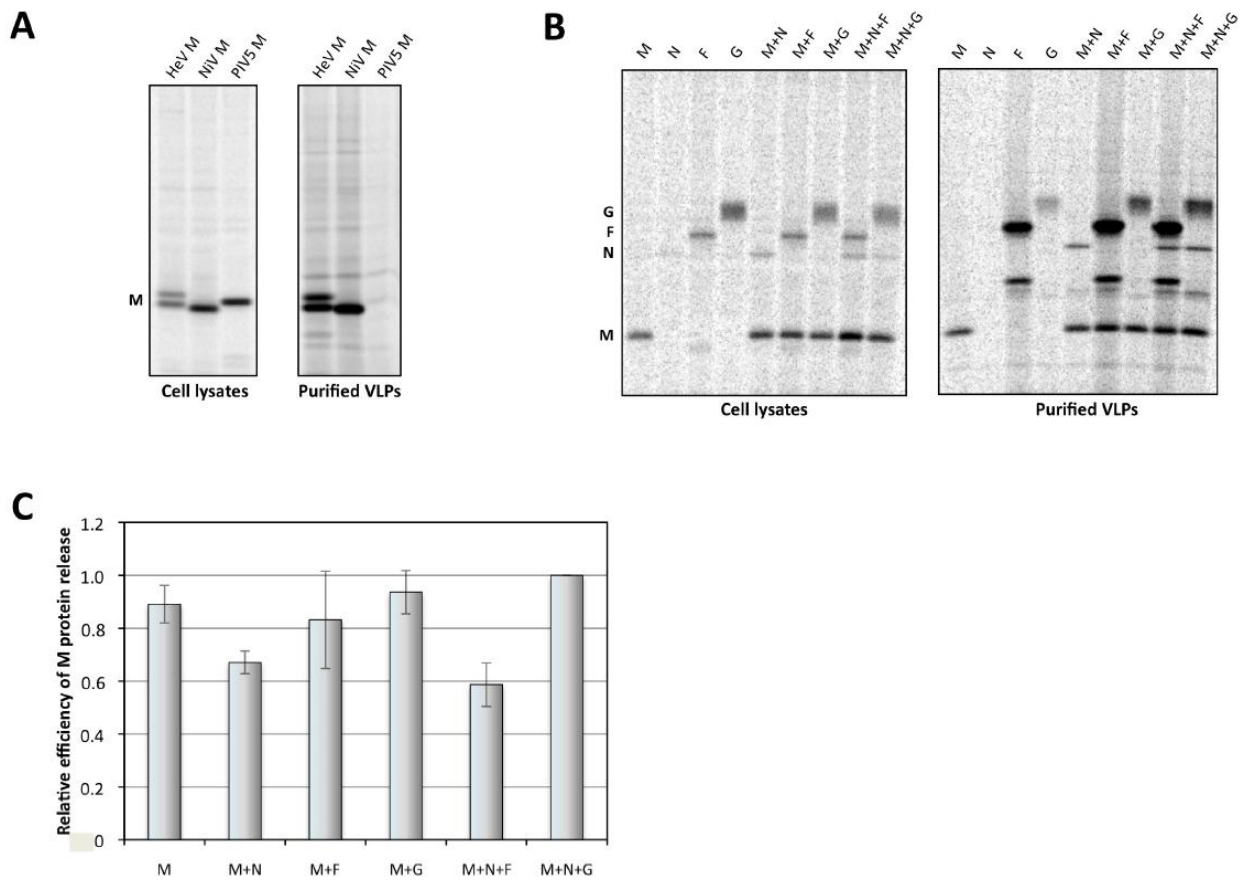


Fig. 3-1 Requirements for Nipah VLP production.

(A) VLPs were produced from HEK293T cells transfected to express HeV, NiV or PIV5 M protein. Viral M proteins were metabolically labeled 24hrs. post transfection with ^{35}S for 18 hrs. The culture media was collected and purified on sucrose floatation and loaded directly onto 10% SDS-PAGE gels. Cell lysates were prepared and immunoprecipitated with either an anti-NiV M protein polyclonal antibody which also cross reacts with HeV M protein or anti-PIV5 M protein monoclonal antibody. Gels were exposed on a storage phosphor screen overnight and scanned on a Fuji FLA-7000 laser scanner. **(B)** Different types of Nipah VLPs were produced by expressing combinations of Nipah virus M, N, F, and G in HEK293T cells. **(C)** Particle production efficiency was calculated as the amount of NiV M protein in purified particles divided by the amount in the cell lysate fraction, normalized to the maximum value obtained in the experiment. Results from three independent experiments were quantified. Error bars indicate standard deviations.

Incorporation of NiV Glycoprotein (G) into Nipah VLPs

Although the NiV G protein did not enhance Nipah VLP production, it was detected at increased levels in the media when co-expressed with NiV M protein (Fig. 3-1B). This incorporation of NiV G protein into Nipah VLPs was further investigated to determine if NiV M and G proteins together formed a single population of VLPs, or if there were multiple distinct populations of NiV M, G, or M + G containing VLPs being produced. Purified Nipah VLPs were sedimented through continuous iodixanol density gradients to determine the density and polypeptide composition of the particles being produced (Fig. 3-2A). The VLPs produced by expressing NiV M protein by itself were less dense with the largest percent of VLPs being found in fraction 5 (1.05g/ml). The density of VLPs produced from NiV M protein by itself could not be compared to NiV G protein particles as the inefficient release of particles caused them to be undetectable in the fractions collected. However, comparison of VLPs produced by expression of NiV M protein by itself with those produced upon co-expression of NiV M, G, and/or N proteins together were found to be far less dense (Fig. 3-2B). The largest percent of VLPs containing NiV M and G proteins were detected lower in the gradient in fraction 9 (1.10g/ml). Taken together, when NiV proteins were co-expressed, they produced a single distinct population of VLPs.

To further examine if the incorporated NiV G protein in VLPs produced particles consistent with paramyxovirus virion morphology, VLPs produced by co-expression of NiV M, N, and G proteins were imaged using transmission electron microscopy (TEM). Purified VLPs were concentrated and visualized by TEM with negative staining. Representative images of VLPs produced from NiV M, N, and G proteins displayed a clear layer of glycoprotein spikes on the outer surface of the particles along with some potentially encapsidated cellular RNA indicated by the black arrow (Fig. 3-2C). Most of

the VLPs observed ranged in size from 100-200nm in diameter; however, some potential VLPs were found to be much larger in size with a diameter of up to 900nm (data not shown). Due to the size of the larger particles, the density of the glycoproteins on the surface was extremely low, making definitive identification difficult. Previous ultrastructural characterization of authentic Nipah virions reported a size range of 40-1900nm in diameter, which was consistent with these observations (Fig. 3-2C) (47, 68).

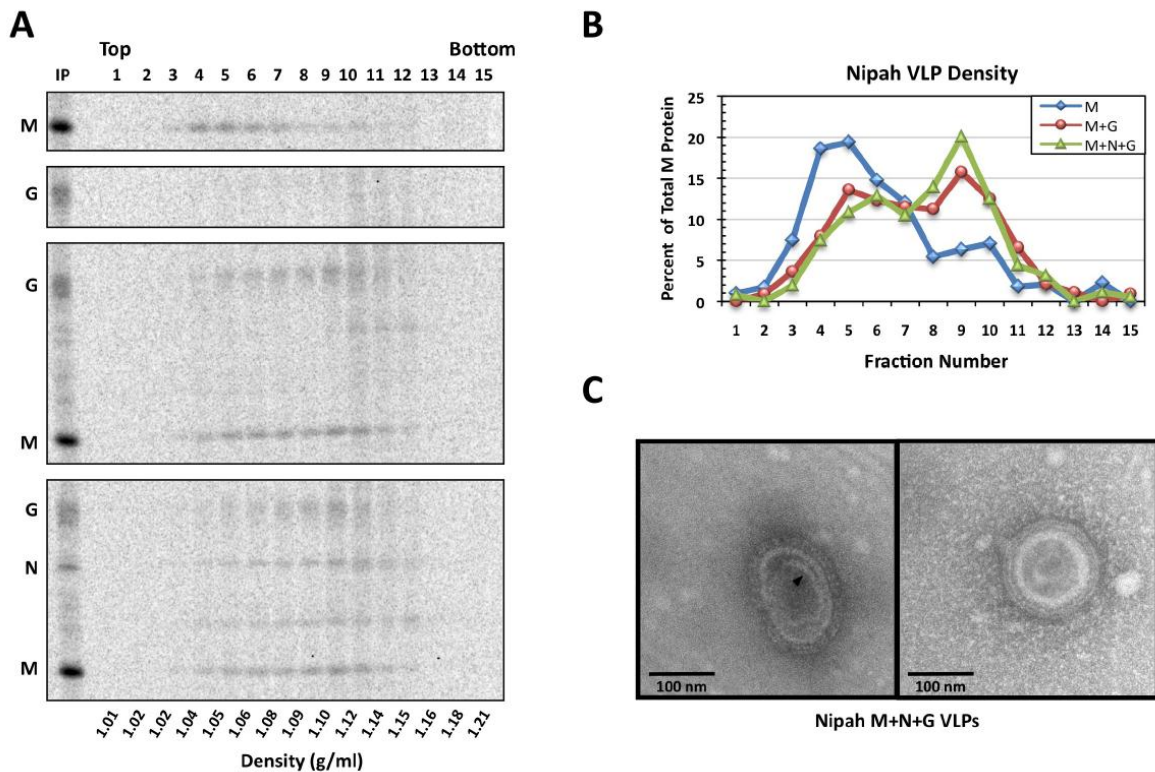


Fig. 3-2 Incorporation of NiV G protein into Nipah VLPs.

(A) Nipah VLPs were produced from transfected HEK293T cells using combinations of NiV M, N, and G expressing plasmids. VLPs were prepared as described previously. Purified VLPs were overlaid onto a continuous iodixanol gradient and centrifuged for 18hrs, after which 15 fractions were collected and weighed before being run on a 10% SDS-PAGE gel. **(B)** The density of the VLPs was quantified for each fraction relative to the total amount of M protein in all 15 fractions collected. NiV G protein alone was not included due to poor efficiency of release. **(C)** Purified VLPs containing NiV M, N, and G protein were passed through a needle 10X before being absorbed onto copper carbon-coated grids and stained with 1% uranyl acetate. TEM images were observed using a JEOL JEM 1200 EX II at 80kV. VLPs observed likely contained cellular RNA encapsidated by NiV N protein, indicated by the black arrow.

NiV C protein does not cooperate with NiV M protein to produce VLPs

Previous studies with SeV have identified a role for C protein in Sendai VLP production (72, 131, 148). NiV also encodes a viral C protein, and to further characterize the requirements of Nipah VLP particle production, NiV C protein was tested for its ability to contribute to efficient Nipah VLP production. In order to investigate this, a C-terminally Flag tagged NiV C protein was co-expressed in HEK293T cells with NiV M protein and VLP production was measured. Unlike SeV C protein, NiV C protein was unable to enhance VLP production (Fig. 3-3A). Although NiV C protein did not enhance Nipah VLP production, it was detected in the media. This observation suggested that NiV C protein might be incorporated into Nipah VLPs.

Characterization of HIV and influenza virus particles have identified a complex polypeptide mixture of viral and cellular proteins, which can be a result of either virus-virus or virus-host protein interactions (17, 138, 139). To test if the incorporation of NiV C protein into Nipah VLPs was a result of a protein-protein interaction between NiV M and C proteins, a co-immunoprecipitation of both overexpressed proteins in HEK293T cells was performed. Immunoprecipitation of either NiV M or C proteins was unable to detect any interaction between NiV M and C proteins. Alternatively, if an interaction is occurring between NiV M and C proteins, it is too transient in nature to be detected using these assay conditions (Fig. 3-3B). These results indicate that, unlike SeV C protein, NiV C protein does not enhance VLP production and that NiV C protein is released into the media via an unknown mechanism.

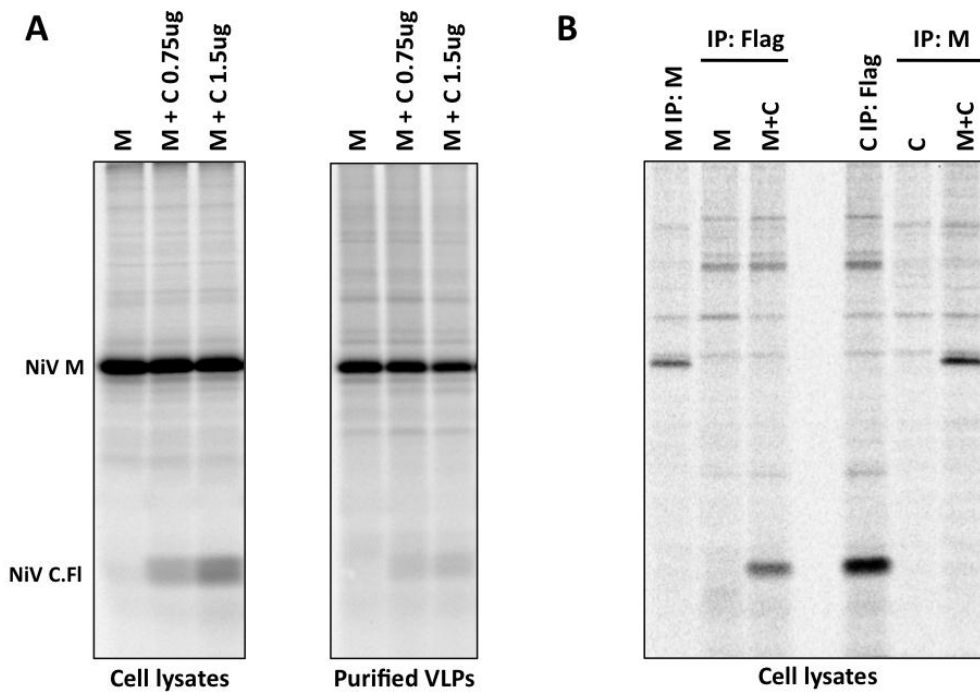


Fig. 3-3 NiV C protein does not contribute to VLP production.

(A) Nipah VLPs were produced from HEK293T cells expressing NiV M protein by itself or co-expressed with NiV C protein using two different plasmid amounts. Viral proteins were metabolically labeled 24hrs. p.t. with ^{35}S for 18hrs. The culture media was collected and purified on sucrose floatation gradients and loaded directly onto 15% SDS-PAGE gels. Cell lysates were prepared and immunoprecipitated with anti-NiV M protein polyclonal antibody and FLAG[®] magnetic affinity resin. Gels were exposed on a storage phosphor screen overnight and scanned using a Fuji FLA-7000 laser scanner. **(B)** NiV M protein was co-expressed with NiV C.FI in HEK293T cells. Cells were pulse labeled 24hrs. p.t. with ^{35}S for 3hrs. Cells were harvested in 0.5% NP-40 lysis buffer and clarified to remove cellular debris. The cell lysates were immunoprecipitated with either NiV M protein polyclonal antibody or FLAG[®] magnetic affinity resin as indicated. Immunoprecipitated cell lysates were washed 3x with lysis buffer and analyzed on a 15% SDS-PAGE gel. Gels were exposed on a storage phosphor screen overnight and scanned on a Fuji FLA-7000 laser scanner.

NiV C protein binds to cellular membranes

The observation that NiV C protein is released into the media independent of an interaction with NiV M protein, along with previous reports that SeV C protein binds with cellular membranes, led to the hypothesis that NiV C protein might also bind to cellular membranes in order to facilitate its incorporation into VLPs (72, 96). Additionally, a sequence comparison of NiV C and SeV C proteins revealed similar highly basic and hydrophobic regions within their N-termini (Fig. 3-4A). This region in NiV C protein is also predicted to form an alpha helix indicative of a potential membrane-binding region. This structure prediction was performed using the Jpred 3 secondary structure prediction server (<http://www.compbio.dundee.ac.uk/jpred>) (22).

To explore this, detergent-free lysates were prepared from HEK293T cells expressing NiV C protein and placed at the bottom of a sucrose density gradient. Samples were ultracentrifuged and serial fractions were collected from the top of the gradient. The top three fractions contained membrane-bound NiV C protein, while the bottom four fractions contained membrane-free protein. Analysis of NiV C protein identified that ~30% of the total NiV C protein detected was bound with cellular membranes (Fig. 3-4C). This level of membrane-binding is similar to that of the NiV M protein (data not shown).

To further investigate the requirements for NiV C protein membrane-binding N-terminal truncations to the first 10 or 20 amino acids and alanine substitutions were made to eliminate the hydrophobic or basic amino acids in this region (Fig. 3-4B). HEK293T cells expressing N-terminally modified NiV C protein were tested to determine the relative contribution of the hydrophobic and basic amino acids in NiV C protein membrane-binding. Removing the first 10 or 20 residues from the N-terminus of NiV C protein completely eliminated NiV C protein membrane-binding (Fig. 3-4C).

Alternatively, mutation of only the five hydrophobic residues to alanine in this N-terminal region also eliminates NiV C protein membrane-binding. However, when the eight basic residues were mutated to alanine, membrane binding was only reduced by 50% as compared to the unmodified NiV C protein (Fig. 3-4C). Together these results suggest a model whereby the hydrophobic and basic residues of NiV C protein together serve as a putative membrane binding sequence.

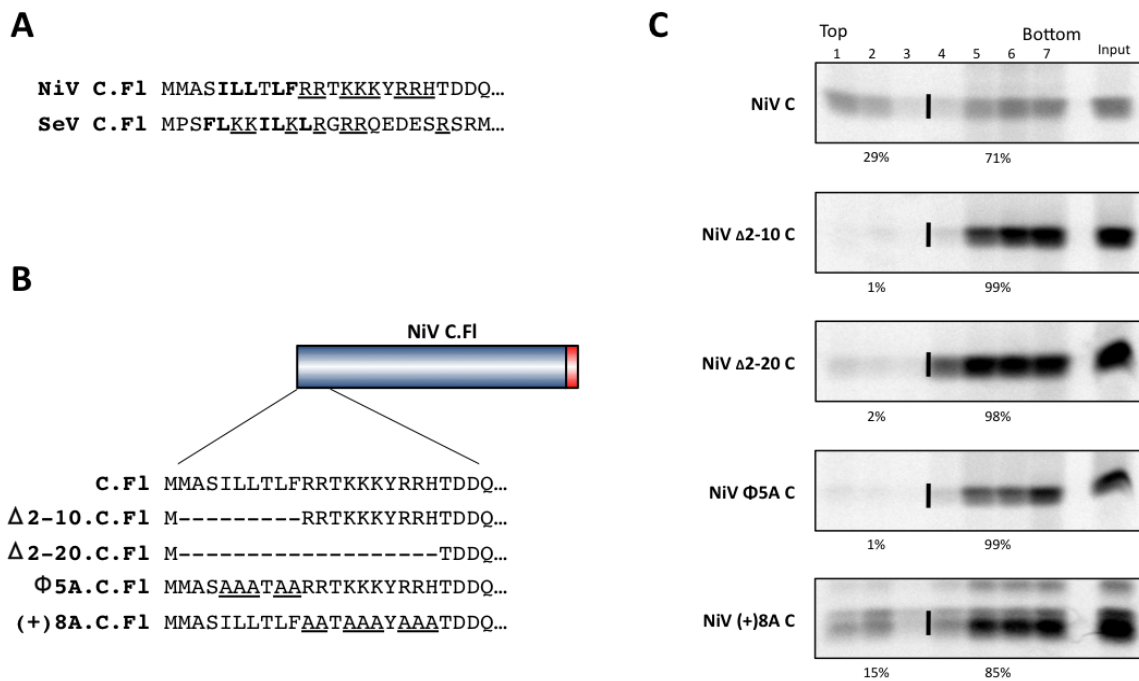


Fig. 3-4 NiV C protein binds to cellular membranes

(A) A sequence alignment between the N-termini of both NiV and SeV C protein demonstrating the similarity in amino acid composition. Hydrophobic amino acids are highlighted in bold and basic amino acids are underlined. (B) A schematic that illustrates the NiV C.F1 protein N-terminal alanine substitutions and deletions tested for membrane-binding. (C) HEK293T cells were transfected with NiV C protein or the described derivatives and pulse labeled with ³⁵S for three hrs. Labeled cells were dounce homogenized in a hypotonic detergent-free lysis buffer, 10% of the total lysate was saved for the input control before and being clarified and loaded at the bottom of a floatation gradient. Samples were centrifuged for 16hrs. in a swinging bucket rotor before fractions were collected and immunoprecipitated with FLAG® magnetic affinity resin. Fractions were resolved on a 15% SDS-PAGE gel. The top three fractions contain membrane-bound protein while the bottom four fractions contain membrane-free protein.

Characterization of a putative membrane targeting sequence

To test if the putative membrane targeting sequence of NiV C protein is sufficient to direct a heterologous protein to bind membranes, it was fused to EGFP (Fig. 3-5A). HEK293T cells expressing NiV₁₋₂₀-EGFP or EGFP were assessed for their ability to bind to cellular membranes using sucrose floatation gradients as described above. Approximately 30% of the total NiV₁₋₂₀-EGFP protein expressed was able to bind with membranes, while no unmodified EGFP was detected in the membrane-bound fractions (Fig. 3-5B). The amount of NiV₁₋₂₀-EGFP detected in the membrane-bound fractions was comparable to what was observed for NiV C protein (Fig. 3-4C). This redirection of EGFP to cellular membranes by the NiV C protein putative membrane targeting sequence supports our finding that this sequence is responsible and sufficient for the membrane-binding of NiV C protein, consistent with previous observations made with SeV C protein (96).

In order to visualize the redirection of EGFP to cellular membrane by the NiV C protein membrane targeting sequence, EGFP, NiV₁₋₂₀-EGFP and a full length NiV C-EGFP fusion were expressed in Vero cells to be examined using fluorescence microscopy. Expression of the unmodified EGFP displayed a diffuse localization pattern throughout the cytoplasm while NiV₁₋₂₀-EGFP was concentrated at perinuclear intracellular membranes (Fig. 3-5C). The localization pattern of the NiV C-EGFP fusion displayed similar perinuclear fluorescence; however, NiV C-EGFP could also be visualized throughout the cytoplasm. These results support the biochemical findings that NiV C protein fusions can redirect EGFP cellular membranes although the function of this cellular localization is not yet understood.

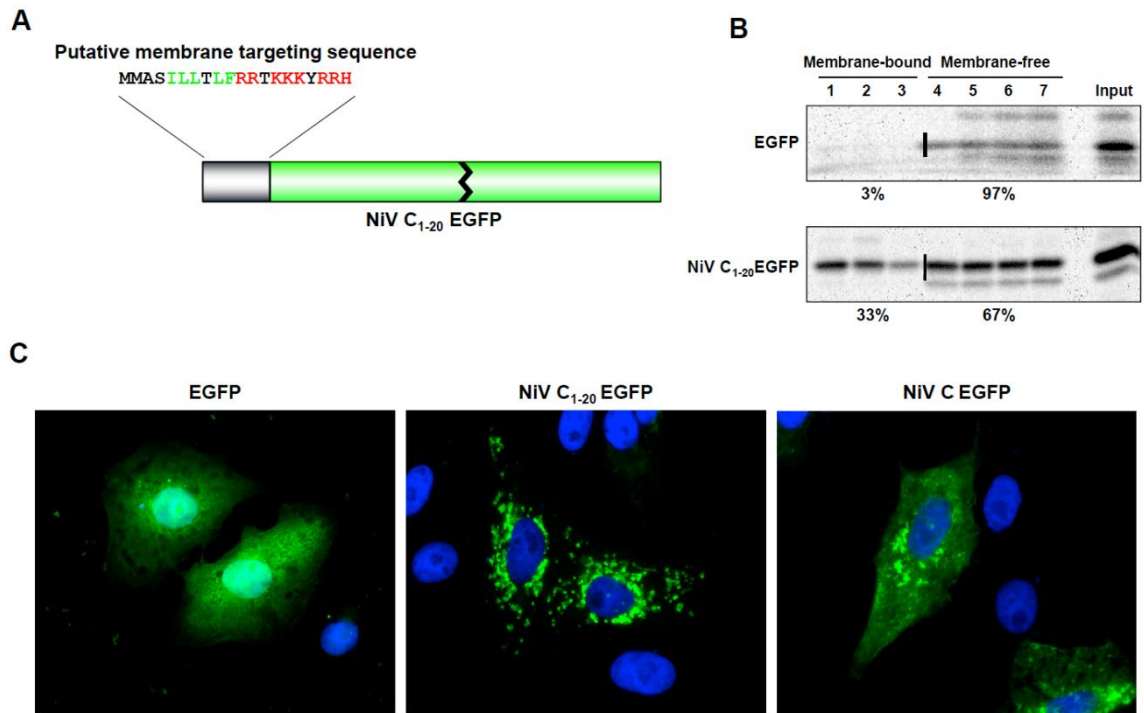


Fig. 3-5 Characterization of a putative membrane targeting sequence

(A) The putative membrane targeting sequence if NiV C protein was appended to the N-terminus of EGFP. **(B)** HEK293T cells transfected to express NiV C₁₋₂₀-EGFP or EGFP were processed as described above to test if the NiV C protein putative membrane targeting sequence could direct EGFP to cellular membranes. **(C)** Vero cells were grown on coverslips and transfected to express NiV C₁₋₂₀-EGFP, NiV C-EGFP or EGFP. Cells were fixed 24hrs. p.t. and permeabilized with 0.1% Triton X-100. Fixed cells were probed with anti-EGFP primary antibody and a Cy3 conjugated secondary antibody to amplify the fluorescence signal before being imaged with a Zeiss Axio Imager.M1 fluorescence microscope.

NiV C protein's release within vesicles depends on membrane-binding

Since there was no detectable interaction between NiV M and C proteins, we tested if a functional membrane targeting sequence was required to detect NiV C protein in the culture media. Nipah VLPs were produced from HEK293T cells transfected to express NiV C protein N-terminal derivatives (Fig. 3-2A) and NiV M protein. The expression of the NiV C protein derivatives that lacked N-terminal hydrophobic amino acid residues were not detected in the culture media when co-expressed with NiV M protein (Fig. 3-6A). Additionally, the detection of NiV C protein in the media was independent of co-expression with NiV M protein (Fig. 3-6B). Given that culture medias are purified using a stringent sucrose floatation step, the protein detected is presumed to be associated with buoyant vesicles. Therefore, it is likely that membrane binding of NiV C protein allows for its incorporation and release within membranous vesicles.

During expression of various NiV C protein derivatives, the NiV (+)8A.C mutant was unexpectedly expressed to levels up to a 30 times greater than any of the other NiV C protein derivatives tested. However, despite such high levels of NiV (+)8A.C protein expression, similar amounts of NiV (+)8A.C protein containing vesicles were released into the culture media as compared to unmodified NiV C protein. Although this observation is not completely understood, it suggests that NiV C protein containing vesicles are released through a regulated mechanism, and that extremely high protein expression levels do not impact this process. Taken together, these results suggest that the release of vesicles containing NiV C protein is regulated process that depends on an intact membrane targeting sequence.

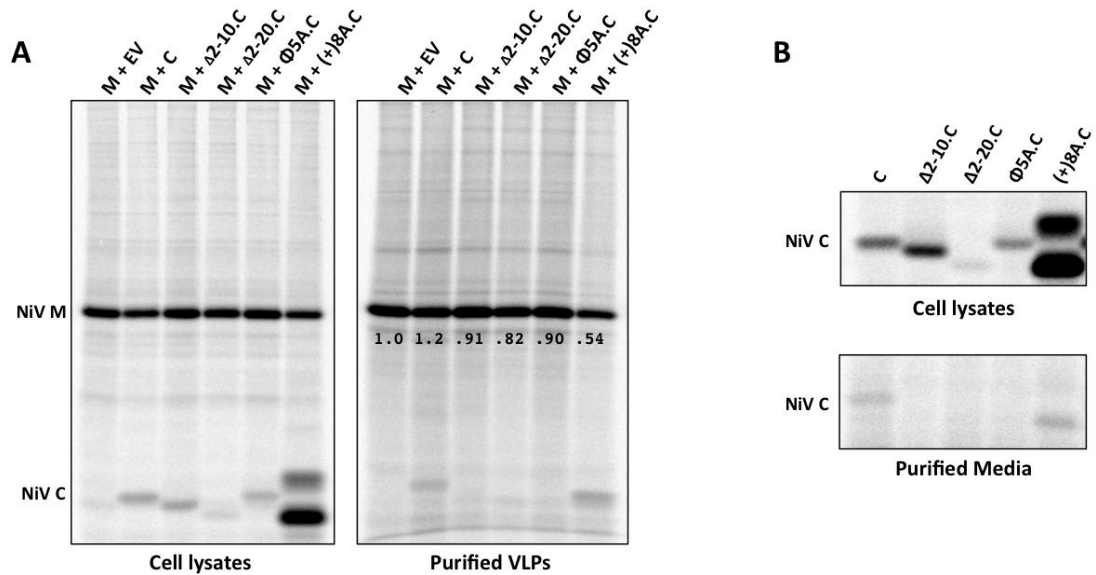


Fig. 3-6 Membrane-binding defective NiV C protein is not detected in the media

(A) Nipah VLP production was measured by expressing combinations of NiV M, C and membrane-binding deficient derivatives in HEK293T cells. Nipah VLPs were purified and cell lysate expression was measured as described previously. Immunoprecipitated cell lysates and purified VLPs were resolved on 15% SDS-PAGE gels and images were generated using Fuji FLA-7000 laser scanner. Particle production efficiency was calculated as the amount of NiV M protein in purified particles divided by the amount in the cell lysate fraction, normalized to the unmodified NiV C protein. **(B)** NiV C protein's ability to release into the media by expressing NiV C or membrane-binding deficient derivatives in HEK293T cells.

An N-terminal cluster of basic amino acids destabilizes NiV C protein

In some cases, viral proteins use basic amino acids to recruit E3 ubiquitin ligases to polyubiquitinate encoded lysine residues for the purposes of protein degradation as a part of the N-end-rule pathway. To investigate if the N-terminal basic amino acids of NiV C protein potentially served as a degradation signal, the protein stability of several NiV C protein mutants was tested. HEK293T cells transfected to express unmodified NiV C protein or several N-terminal derivatives (Fig. 3-7A) were pulse labeled with ^{35}S for 15 minutes. Following the label period, culture media was exchanged for unlabeled media and cells were harvested at 0, 30, or 60 minutes later. Additionally, the proteasome inhibitor MG-132 was included to prevent any NiV C protein proteasome-dependent degradation. The unmodified NiV C protein was found to be particularly unstable with a half-life of only 30 minutes (Fig. 3-7B). This instability was also proteasome-dependent, as treatment with MG-132 completely prevented protein degradation. The elimination of the hydrophobic region required for membrane binding had no effect on NiV C protein stability. However, the mutation of all eight basic amino acids in NiV (+)8A.C protein stabilized the protein. Although the implications are not fully understood, the NiV (+)5A.C protein, which maintained a cluster of three lysine residues, was observed to further decrease instability compared with the unmodified NiV C protein. Together, these results are consistent with the possibility that the basic amino acids in the NiV C protein N-terminus may function as a degradation signal in the N-end-rule pathway.

A

C.FI MMAS ILLT LFRRT KKKYRRHTDDQ...
 Φ5A.C.FI MMAS AAAATAARRT KKKYRRHTDDQ...
 (+)8A.C.FI MMAS ILLT LFAATAAAAYAAATDDQ...
 (+)5A.C.FI MMAS ILLT LFAAT KKKYAAATDDQ...
 K14-16A.C.FI MMAS ILLT LFRRT AAAAYRRHTDDQ...

B

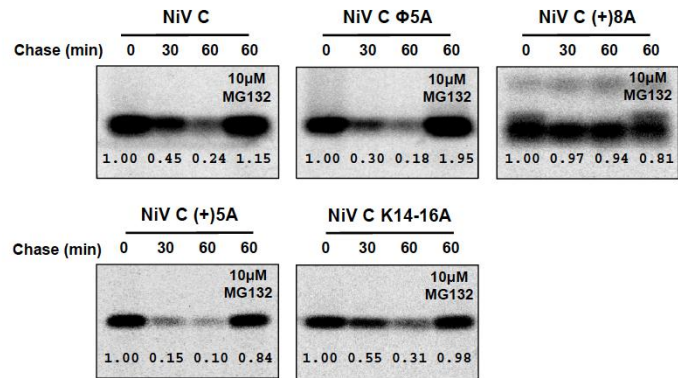


Fig. 3-7 A N-terminal cluster of basic amino acids destabilizes NiV C protein

(A) Schematic showing the alanine substitutions made to the N-terminus of NiV C protein. **(B)** HEK293T cells expressing NiV C protein N-terminal derivatives were labeled with ³⁵S for 15 min. before media was exchanged with chase media lacking ³⁵S and containing either DMSO or 10 μM of MG-132. Cells were harvested at chase periods of 0, 30, or 60 min. and immunoprecipitated with FLAG® magnetic affinity resin. Immunoprecipitated samples were resolved on 15% SDS-PAGE gels and imaged using a Fuji FLA-7000 laser scanner. Protein levels were quantified for each sample and normalized relative to the amount of NiV C protein present at the 0 min. chase period.

NiV C protein is ubiquitinated at multiple lysine residues

Typically, proteins that undergo proteasome-dependent degradation are polyubiquitinated prior to degradation. To determine the ubiquitination status of NiV C protein, HA-tagged ubiquitin (HA-Ub) and/or NiV C protein were expressed in HEK293T cells. The overexpression of HA-Ub is often used to increase the fraction of ubiquitinated protein species to allow for their detection; however, there was concern that this may lead to increased degradation of NiV C protein. To address this concern, duplicate samples were treated with MG-132 to prevent proteasome-dependent degradation. Cells were lysed using a strong detergent to disrupt any potential protein-protein interactions without affecting the covalent attachment of ubiquitin to NiV C protein. Cell lysates were immunoprecipitated with anti-flag resin and samples were subjected to immunoblotting. The ubiquitinated species were detected with an anti-ubiquitin antibody (VU-1). However, NiV C protein could not be detected by anti-flag antibody so the whole protein stain, Sypro orange, was used to detect C protein.

Several higher molecular weight species were detected above NiV C protein with or without co-expressed HA-Ub (Fig. 3-8A). These species were also not detected in samples only expressing HA-Ub. The polypeptide composition of likely ubiquitinated NiV C protein species resembled a protein ladder with four major bands and a higher molecular weight smear consistent with most polyubiquitinated proteins. The major NiV C protein species were labeled C0, C1, C2, C3, or C4 where C0 represents unmodified NiV C protein and C1-C4 represent the attachment of between one and four ubiquitin molecules. Each of these major bands is approximately separated by 8.5kDa, the molecular weight of a single ubiquitin molecule.

Although it was clear that species C1-C4 represented the addition of several ubiquitin molecules, it was still unclear if this was a result of monoubiquitination at

several ubiquitin attachment sites along NiV C protein or if polyubiquitination was occurring at just a single site. To address this question, HA-Ub KO, which has all of its internal lysine residues mutated to arginine, was overexpressed with NiV C protein. This mutant prevents ubiquitin chain extension and allows for identification of the total number of attached ubiquitin molecules. The ubiquitination pattern of NiV C protein when co-expressed with HA-Ub KO was largely unchanged except for a reduction in the higher molecular smear seen with co-expression of HA-Ub (Fig. 3-8B). This result suggested that at least four lysine residues serve as sites of ubiquitin attachment. However, it cannot be distinguished if these sites are solely monoubiquitinated or if they serve as a platform for ubiquitin chain extension.

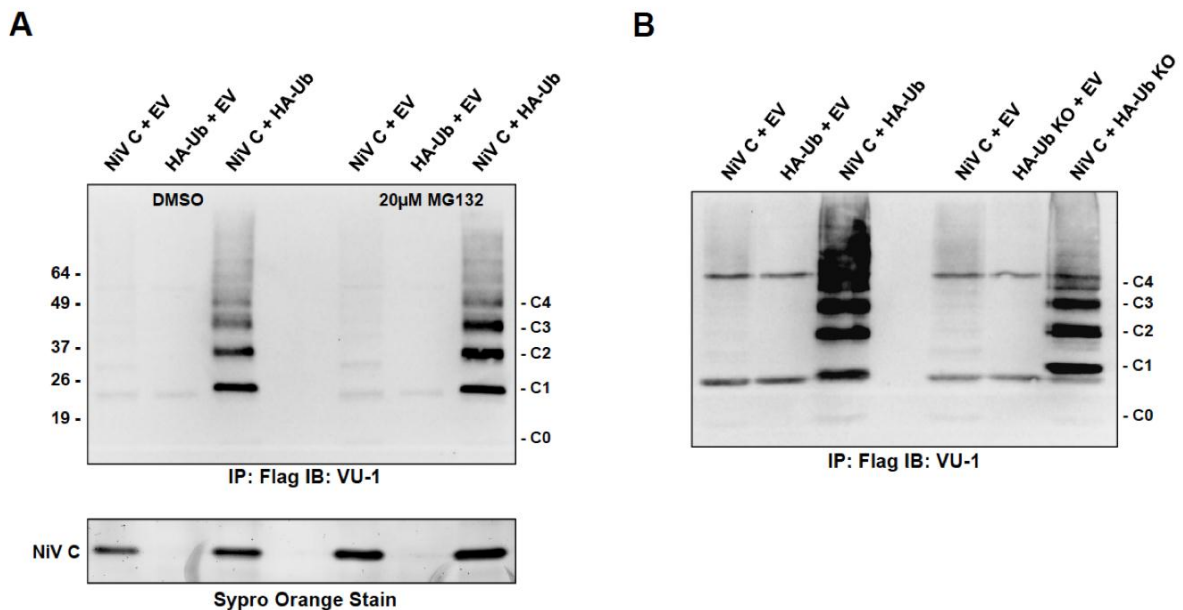


Fig. 3-8 NiV C protein is ubiquitinated on at least four lysine residues

(A) HEK293T cells were transfected to express the indicated combinations of NiV C, and HA-Ub in duplicate. 21h. p.t. cells were treated with either DMSO or 20µM MG-132 for 3h. then cells were harvested and lysed in RIPA buffer. Cell lysates were clarified and immunoprecipitated with FLAG[®] magnetic affinity resin for 3h. Samples were loaded in duplicated onto 15% SDS-PAGE gels and immunoblotted with a α -ubiquitin antibody (VU-1) or stained with Sypro Orange to detect NiV C protein levels. Protein size marker indicates the apparent molecular weight (kDa) for estimate the size of NiV C protein species labeled C0, C1, C2, C3, and C4. **(B)** NiV C protein ubiquitination was measured as described above except HA-UB KO (ubiquitin lacking internal lysine residues) was used to prevent chain extension following NiV C protein ubiquitination.

Mutation of C-terminal lysine residues does not impair NiV C protein ubiquitination

In an effort to identify specific sites of ubiquitin attachment, cumulative lysine to arginine point mutations were made to NiV C protein. In total, there are 14 lysine residues encoded by NiV C protein, K1-K14, any of which could serve as sites for ubiquitin attachment (Fig. 3-9A). To identify likely sites of ubiquitin attachment, a bioinformatics tool that predicts sites of ubiquitin attachment identified a cluster of lysine residues near the C-terminus of NiV C protein as being potential site of ubiquitin attachment (Fig. 3-9B). This predictive algorithm is based on previous work defining predicted ubiquitination sites using the Bayesian Discriminant Method (11). Cumulative lysine point mutations of NiV C protein were constructed from K14 to K11. The ubiquitination profile of these NiV C protein mutants were obtained as previously described above. However, despite that K11-14 were predicted to be sites of ubiquitin attachment, there was no detectable reduction in any of the NiV C protein ubiquitinated species (Fig. 3-9C).

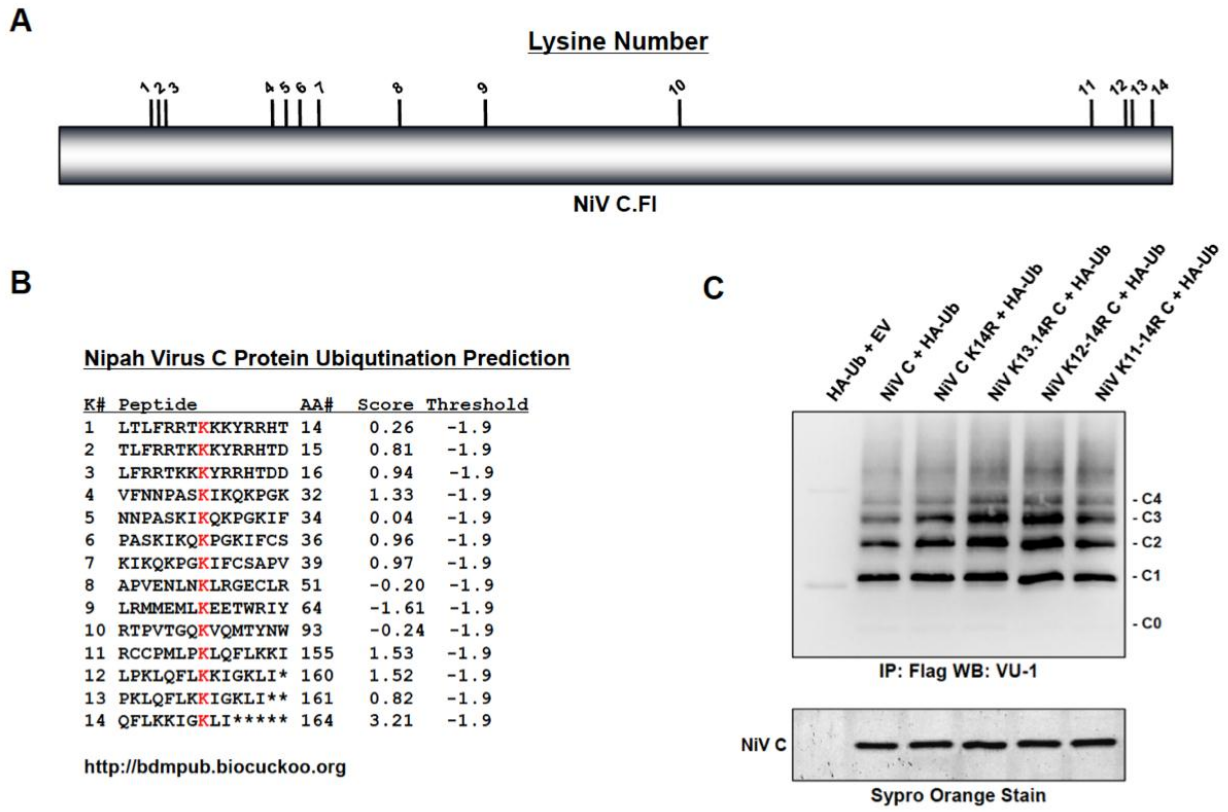


Fig. 3-9 Mutation of C-terminal lysine residues does not impair NiV C protein ubiquitination
(A) To scale schematic depicting all 14 internal lysine residues in NiV C protein numbered K1 thru K14. **(B)** The predicted NiV C protein sites of ubiquitin attachment based on the Bayesian Discriminant Method at <http://bdmpub.biocuckoo.org>. Based on this prediction the high scores (>1.5) in the C-terminal cluster, K11-K14, was targeted first for Lys to Arg mutation. **(C)** NiV C proteins with cumulative C-terminal Lys to Arg mutations were expressed in HEK293T cells with HA-Ub. The ubiquitination status of these mutants was tested as described above. Ubiquitinated NiV C protein was detected using α -ubiquitin antibody and NiV C protein levels were detected using sypro orange staining.

Mutation of all lysine residues in NiV C protein eliminates ubiquitination and improves stability

Since a direct approach to the identification of sites of ubiquitin attachment to NiV C protein was unsuccessful, a more comprehensive strategy was employed. To further investigate which NiV C protein lysine residue/s are the primary sites of ubiquitin attachment, sequential N-terminal lysine to arginine mutations were made to the NiV C.K11-14R mutant. The ubiquitination status of these additional NiV C protein mutants was tested as compared to the unmodified NiV C protein. While there was a substantial reduction in all of the major ubiquitination bands upon additional mutation of the first three lysine residues, only complete removal of all 14 NiV C protein lysine residues resulted in a dramatic loss in NiV C protein ubiquitination (Fig. 3-10A). These results suggest that although there may be some preferential ubiquitination sites on NiV C protein there is no single site or region required for ubiquitination.

The complete lysine knockout NiV C protein was then tested to determine the impact of a lack of ubiquitination on protein stability. Surprisingly, NiV C protein lacking all lysine residues was not completely stable as observed with the NiV (+)8A.C protein mutant. The half-life of NiV C.K1-14R mutant was only twice that of the unmodified NiV C protein (Fig. 3-10B). However, inhibition of the proteasome with MG-132 was unable to prevent the NiV C.K1-14R from being partially degraded with only 20% more protein remaining as compared to the untreated time point. This result suggests that some of the protein degradation observed in the NiV C.K1-14R mutant is proteasome-independent and unrelated to its ubiquitination.

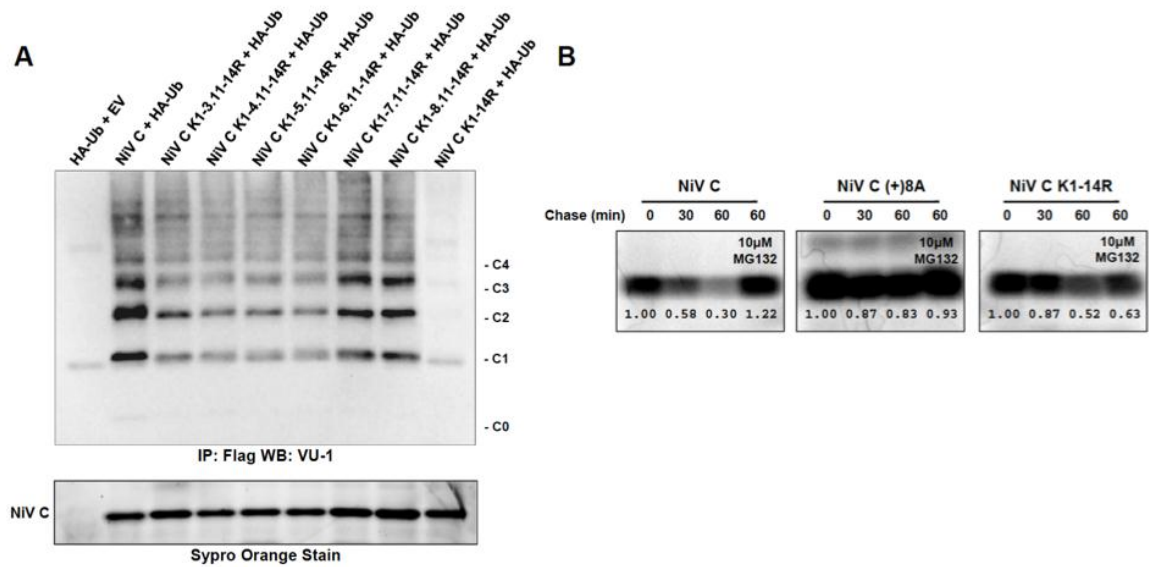


Fig. 3-10 Only the complete knockout of all lysine residues encoded by NiV C protein eliminates ubiquitination and improves stability

(A) HEK293T cells expressing cumulative mutations to all encoded Lys residues were tested for ubiquitination as described above. Ubiquitinated NiV C protein was detected using α -ubiquitin antibody and NiV C protein levels were detected using sypro orange staining. (B) The stability NiV C protein or derivatives was measured as described above. Briefly, HEK293T cells expressing NiV C, C (+)8A, or C K1-14R mutants were pulse labeled with ^{35}S for 15 min. and chased with 2% DMEM lacking label. Cells were harvested at chase periods of 0, 30, 60 mins. and immunoprecipitated with FLAG[®] magnetic affinity resin. Samples were resolved on 15% SDS-PAGE gels.

Working model of the molecular characteristics of NiV C protein

These data suggest a model where NiV C protein is ubiquitinated by one or more cellular ubiquitin ligases. This ubiquitination event likely results in the production of both mono- and poly- ubiquitinated forms of NiV C protein. The polyubiquitination of NiV C protein normally directs the proteasome-dependent degradation NiV C protein. The degradation of NiV C protein does not require an intact membrane targeting sequence, as degradation still occurs even upon removal of the hydrophobic N-terminal amino acid residues required for membrane binding (Fig. 3-11). However, when ubiquitination is prevented through mutation of all 14 lysine amino acid residues NiV C protein does not undergo proteasome-dependent degradation. Alternatively, proteasome-dependent degradation can also be prevented through mutation of N-terminal basic amino acid residues, although the mechanism that prevents degradation is unknown.

These data presented here also suggest that the ubiquitination of NiV C protein is important for its secretion into the media. This requirement is consistent with there being monoubiquitinated forms of NiV C protein, as monoubiquitination of viral matrix proteins has been shown to be required for the budding of some paramyxoviruses viruses (55, 162). These monoubiquitinated forms of NiV C protein are hypothesized to secrete either via the MVB exosomal pathway or directly from the plasma membrane of the cell, either pathway may also require ESCRT machinery (Fig. 3-11). Regardless of the mechanism of secretion, NiV C protein that is unable to be ubiquitinated or unable to bind membranes does not release into the media. However, mutation of the basic N-terminal amino acid residues does not prevent NiV C protein release and despite an increase in protein stability this NiV C protein derivative still releases into the media to similar levels as compared to unmodified NiV C protein.

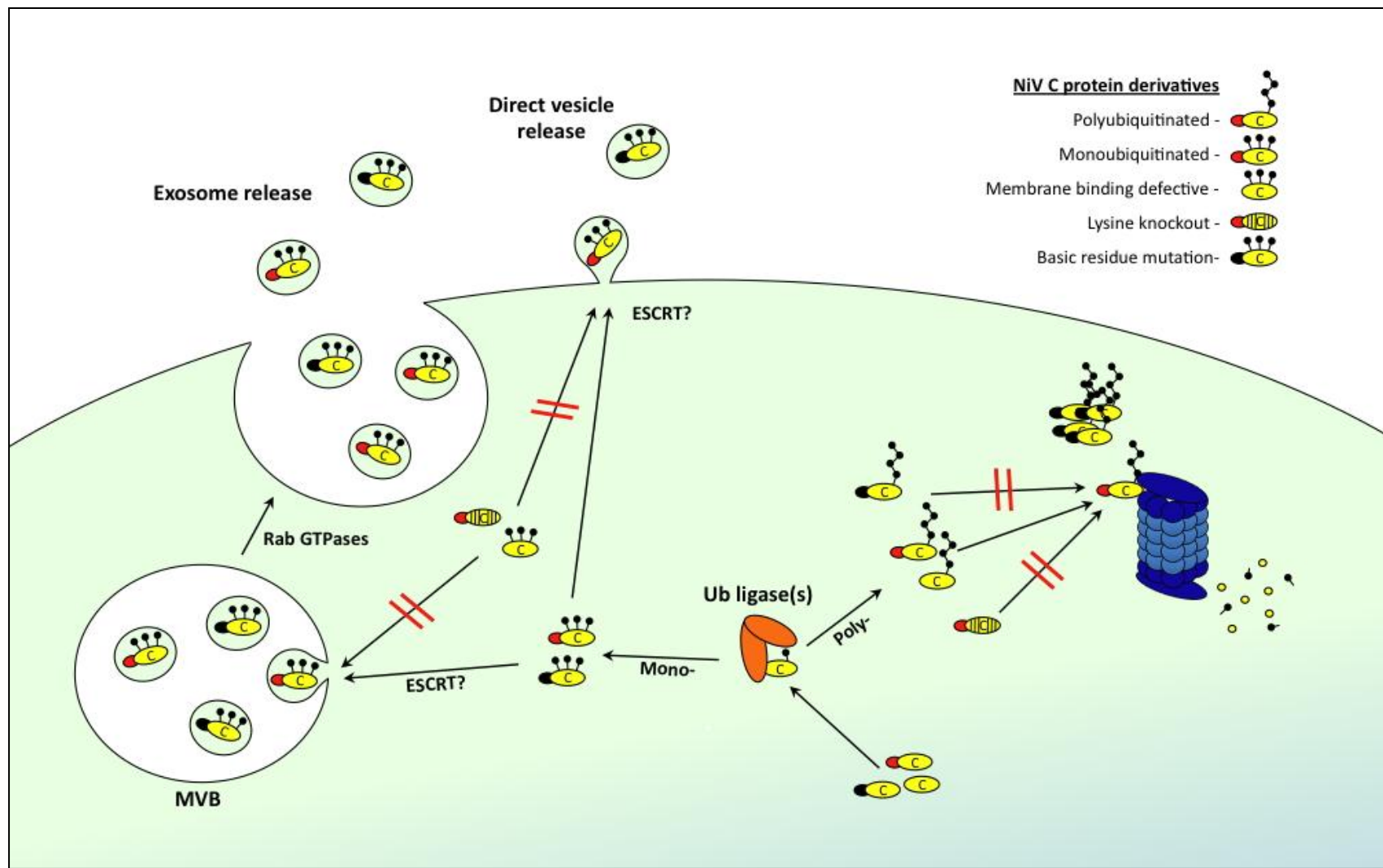


Fig. 3-11 Working model for the molecular characteristics of NiV C protein

This is a working model that diagrams the potential fates of many NiV C protein derivatives. NiV C protein and derivatives are initially directed to ubiquitin ligase(s) where they are either mono- or poly- ubiquitinated. Polyubiquitinated NiV C protein is then directed to the proteasome for protein degradation except the NiV (+)8A.C protein, which accumulates to high levels within the cell. Monoubiquitinated NiV C protein that contains an intact membrane targeting sequence is secreted either through an MVB exosomal pathway or directly from the plasma membrane.

3.3 Discussion

In this study we have confirmed that NiV M protein is the only major contributor to Nipah VLP production, and we defined several molecular characteristics of NiV C protein that are likely to be important for NiV C protein function during virus infection. We have identified NiV C protein as a membrane-binding protein and identified an N-terminal membrane targeting sequence that is sufficient to target EGFP to cellular membranes. Additionally, NiV C protein was found to be particularly unstable and this instability was dependent on the ubiquitin-proteasome pathway. NiV C protein was also found to be highly ubiquitinated and that this ubiquitination could only be eliminated by removal of all 14 encoded lysine residues. The stability of the resulting mutant was partially improved; however, some proteasome-independent degradation was detected. Interestingly, mutating eight N-terminal basic amino acids could also stabilize NiV C protein; however, this observation not fully understood. Here, the molecular characterization of NiV C protein has identified several properties that may be integrally linked to NiV C protein's function and provided a basis for future study aimed at defining the relative importance of these characteristics during virus infection.

Previous findings defining the requirements for Nipah VLP production suggests that expression of viral glycoproteins are not required for efficient VLP production (19, 117). However, other paramyxoviruses require cooperation between several viral proteins for efficient VLP production (87, 136, 148). Due to the different VLP production requirements among paramyxoviruses and differences in our method used to purify VLPs, we wanted to confirm the previously established requirements for Nipah VLP production. We were able to confirm that only NiV M protein expression was required for efficient Nipah VLP production. However, the ability to efficiently produce VLPs with only M protein is not unique to NiV and HeV. Newcastle disease virus (NDV) and

human parainfluenza virus type 1 (hPIV1) are also able to efficiently produce VLPs upon expression of M protein by itself (23, 113).

During investigation of the Nipah VLP production requirements, it was observed that the NiV G protein released into the media independent of NiV M protein expression. Although this is not uncommon for paramyxovirus glycoproteins, this finding raised the possibility that there were potentially two populations of Nipah VLPs being produced. A sedimentation gradient analysis of Nipah VLPs was able to confirm that NiV G protein is specifically incorporated into a single population of Nipah VLPs when NiV M and G protein are expressed together. This likely occurs through a direct interaction between NiV M protein and the cytoplasmic tail of NiV G protein. The importance of this interaction was previously demonstrated using recombinant PIV5 harboring truncations in the cytoplasmic tail of HN protein. Deletion of the cytoplasmic tail of PIV5 HN protein disrupted the M-glycoprotein interaction and prevented accumulation of PIV5 M protein at sites of virus assembly (134). Thus, although NiV G protein does not enhance Nipah VLP production, its incorporation into particles is a critical step in the production of infectious virions.

Our additional characterization of Nipah VLPs found the morphology and size range of VLPs containing NiV M, N, and G proteins to be consistent with previous ultrastructural findings of authentic virions. However, the wide variation in size of Nipah VLPs and virions is not typical among other paramyxoviruses, which are normally ~100nm in diameter (47, 68, 83). Although extremely large Nipah VLPs and virions (>1 μ m in diameter) can be produced in tissue culture, they are presumed to be less infectious because they contain a less densely packed glycoprotein layer compared to smaller particles. These large NiV particles also may not be produced during infection in native fruit bat populations; this large particle morphology could be a result of infections being performed in human tissue culture cell lines. To address this concern, tissue

culture cell lines derived from bats are currently in development. Since many emerging viruses including NiV, HeV, Australian bat lyssavirus (ABLV) and Menangle virus all originate from bats, these cell lines will provide useful tools for exploring the replication of these viruses in their natural host species. These studies may also provide insight into why bat viruses tend to increase in pathogenicity upon crossing species-specific barriers.

Several previous reports have indicated that SeV C protein is able to enhance Sendai VLP production when transfected in mammalian cells (72, 131, 148). To further our understanding of the requirements for Nipah VLP production, we investigated whether the NiV C protein could enhance VLP production. Unlike SeV C protein, we found that NiV C protein could not enhance Nipah VLP production although it was detected in the culture media. Surprisingly, the detection of NiV C protein in the culture media was independent of NiV M protein expression. One possible mechanism of NiV C protein release into the media is within a cellular vesicle such as an exosome. The entry of NiV C protein into exosomes may be dependent on protein-protein interactions with exosomal components.

One such component, AIP1, is commonly associated with the release of exosomes and the incorporation of exosomal cargo (3, 67). AIP1 also has a well-characterized role in enveloped virus budding as a component of the ESCRT machinery (32, 94, 146). Interestingly, NiV C protein encodes the same AIP1-binding motif (YP(X)_nL) used for the ESCRT-dependent virus budding and incorporation of exosomal cargo. We found that mutation of the putative AIP1-binding motif in NiV C protein or disruption of the ESCRT machinery with a dominant negative form of VPS4 prevented NiV C protein from releasing into the media (data not shown). Although this evidence suggests that there may be a protein-protein interaction occurring between NiV C protein and AIP1 we have not confirmed an interaction in mammalian cells (data not shown). If

any interaction is occurring between NiV C protein and AIP1, it may be more transient in nature or undetectable with our binding conditions.

The release of microvesicles such as exosomes from cells commonly serves as a type of intercellular communication (Reviewed in 100). Numerous reports have observed that the release of microvesicles containing viral proteins can contribute to viral pathogenesis (Reviewed in 102). For example during HIV-1 infection, exosomes are secreted that contain the viral Nef protein (86). In this case, Nef protein containing exosomes behave as a Trojan horse, entering uninfected peripheral blood lymphocytes (PBLs) to induce cell death (86). These findings raise the possibility that NiV C protein may also be secreted within a microvesicle such as an exosome. With the many described functions of NiV C protein including the inhibition of inflammatory cytokine production and IFN-signaling, transfer of NiV C protein into nearby cells via exosomes would represent a clear benefit for viral replication and spread.

SeV C protein, which has functional similarities to NiV C protein, is able to bind cellular membranes through a N-terminal membrane targeting sequence (96). A similar N-terminal membrane targeting sequence (**MMASILLTLFRRTK**KKYRRH...; hydrophobic residues are highlighted in bold; basic residues are underlined) directs NiV C protein to perinuclear membranes. However, unlike some viral proteins that bind membranes, the NiV C protein membrane targeting sequence does not contain any potential sites of palmitoylation or myristoylation to facilitate membrane binding. Instead, it requires a cluster of hydrophobic and basic residues for optimal membrane binding. Additionally, the disruption of this membrane targeting sequence prevents NiV C protein from releasing into the media. This dependence on membrane binding suggests that release of NiV C protein into the media is occurring in a relatively specific manner and is not likely the result of inclusion into apoptotic blebs from dying cells. Additional support for this specificity comes from our finding that the NiV (+)8A.C protein mutant releases

similar levels of protein into the media as compared with unmodified NiV C protein, despite being expressed at much higher steady state levels.

Further investigation into the elevated NiV (+)8A.C protein expression levels found it to be a stabilized derivative of the normally unstable NiV C protein. Viral protein instability is not uncommon; work with SeV C protein has identified a link between SeV C protein's instability and STAT1 degradation (42). Additionally, proteasome-dependent degradation has also been previously reported for the Sindbis viral polymerase and HIV-1 integrase (27, 108). The degradation of these proteins requires an N-terminal basic residue, which functions as a degradation signal recognized by the N-end-rule pathway. This pathway can recognize both basic and hydrophobic amino acids, type 1 or type 2 N-degrons, as protein degradation signals. These N-degron sequences are responsible for recruitment of E3 ubiquitin ligases termed N-recognins, such as UBR1, UBR2, UBR4, and UBR5 (144, 145). Since, the mutation of the N-terminal basic amino acids in the NiV (+)8A.C protein mutant resulted in dramatic protein stabilization, this region within NiV C protein may serve as an internal degron for the recruitment of an E3 ubiquitin ligase by NiV C protein. Moreover, given the similarities between NiV and SeV C proteins, the instability of NiV C protein may function to degrade components of the host cellular IFN-signaling pathway.

Further characterization of NiV C protein found its instability to be dependent on the polyubiquitination of lysine residues. Ubiquitination of substrate proteins is often the result of a stochastic choice by the ubiquitin ligase as to which lysine residue ubiquitin will be attached to (156). This held true for NiV C protein, as no single lysine residue proved to be critical for ubiquitination. The only way to severely reduce NiV C protein ubiquitination and restore some protein stability was to completely remove all of ubiquitin attachment sites within the protein. However, during this analysis we could not exclude the possibility that NiV C protein may also be monoubiquitinated. Two findings

suggested that monoubiquitination was occurring on NiV C protein. 1) The complete removal of lysine residues also prevented NiV C protein's release into the media (data not shown). Similarly, many other enveloped virus gag or M proteins require monoubiquitination for proper trafficking and budding of virus particles (55, 61, 143, 162). 2) The NiV (+)8A.C protein mutant was still ubiquitinated and released into the media despite not being degraded (data not shown). Although it is unclear why this derivative of NiV C protein is not being degraded, the presence of ubiquitinated species suggests that there is another role for the ubiquitination of NiV C protein. Based on these data we suggest a model where the polyubiquitination of NiV C protein directs its proteasome-dependent degradation with a subset of NiV C protein being monoubiquitinated to direct protein trafficking to sites of exosome assembly and release. However, further investigation of these findings is required to better understand how these molecular characteristics of NiV C protein direct its function during virus infections.

Chapter 4

Identification of Serine-rich Acidic Host Factors that Bind Henipavirus Matrix Proteins

4.1 Introduction

In chapter 3 we investigated the role of several viral proteins in *Henipavirus* assembly and budding of virus-like particles (VLPs). Here, we have extended this work to identify host factors that may contribute to the release of VLPs by *Henipavirus* matrix (M) proteins. To this end we set out to develop small peptide inhibitors of *Henipavirus* particle production.

Small peptide inhibitors of enveloped virus budding

Overexpression of small peptides that bind to viral M or gag proteins can often inhibit enveloped virus assembly and budding. Typically, this inhibition disrupts the recruitment of host factors required for proper viral protein function. For example, overexpression of the TSG-5' or V-domain peptides derived from the endosomal sorting complex required for transport (ESCRT) components, TSG101 or AIP1 respectively, inhibits HIV-1 gag protein function resulting in a 5-fold reduction in virus particle release in infected cells (30, 109). Furthermore, studies with another retrovirus, equine infectious anemia virus (EIAV), suggest that overexpression of these inhibitory peptides requires the presence of specific TSG101 or AIP1 binding motifs within the viral protein. Exchanging the TSG101 binding motif with the AIP1 binding motif for renders EIAV insensitive to overexpression of TSG-5' (94). These findings demonstrate how small inhibitory peptides can be used to specifically inhibit viral protein function with minimal off-target effects. Based on these findings, work is currently underway to create small molecule inhibitors that block the HIV-1 gag-TSG101 interaction using detailed structural

maps of this protein-protein interaction (69, 124, 151). The further development of this type of small molecule inhibitors may lead to the production of a novel drug used to improve the current treatment for HIV infected individuals.

Although paramyxoviruses generally lack well-characterized ESCRT binding motifs, virus budding can still be inhibited through overexpression of small inhibitory peptides. One such peptide was derived from the host protein angiomin-like protein 1 (AMOTL1) following yeast 2-hybrid screening of parainfluenza virus type 5 (PIV5) M protein (119). This peptide, m-AMOTL1, was developed from mapping studies of the M-AMOTL1 binding region. The overexpression of m-AMOTL1 dramatically reduces PIV5 VLP production by 10-fold (119). In addition to inhibiting PIV5 VLPs, AMOTL1 derived inhibitory peptides can also be used to reduce the production of virus particles produced from PIV5 infected cells (119). Together, these studies demonstrate how identification of inhibitory peptides can provide insight into host factors required for virus particle production, and they represent potentially attractive targets for antiviral drug design. In this work we set out to identify host factors that are critical for *Henipavirus* M protein function and to develop small peptide inhibitors of assembly and budding.

Yeast 2-hybrid and co-affinity purification screening

Screening for virus-host protein interactions is a time-tested method for identification of novel cellular factors that can play an integral role in many aspects of virus life cycles. A wide variety of strategies have been used to identify these virus-host protein interactions (Reviewed in 104), and yeast 2-hybrid screening has been used extensively for this purpose. Use of this system has resulted in the establishment of viral interactome networks for dengue virus (DENV), West Nile virus (WNV), severe acute respiratory syndrome (SARS), human immunodeficiency virus type 1 (HIV-1), and several herpesviruses (9, 37, 75, 84, 159). This system exploits the fact that many

transcription factors can be separated into a DNA-binding domain (DBD) and an activation domain (AD) that are fused with either the bait or prey protein of interest. When an interaction occurs between both bait and prey proteins, the transcription factor is reconstituted leading to activation of reporter genes that allow for selection of candidate binding proteins.

In addition to yeast 2-hybrid screening, co-affinity purification has been used to identify the composition of virus particles and to identify virus-virus or virus-host protein interactions. Typically, the protein of interest is fused with an affinity tag such as a Strep, 6-His or GST to allow for protein purification. Co-affinity purification of a tagged viral protein can then be used to screen for novel virus-host protein interactions by identifying the co-purified polypeptides using mass spectrometry. However, purification of viral proteins from cell extracts can result in the identification of a complex mixture of co-purified host proteins. For this reason, identification of the direct protein-protein interactions can be more difficult as compared to yeast 2-hybrid screening. Despite this difficulty, the numerous host proteins identified can provide useful insight into particular cellular machinery required for viral protein function. Here, both co-affinity purification and yeast 2-hybrid screens were used to identify *Henipavirus* M-interacting proteins.

4.2 Results

Construction of Viable Bait Constructs and cDNA Library Screening

To identify host proteins that interact with *Henipavirus* M protein three baits were constructed which encoded the entire M protein for Nipah virus (NiV) and Hendra virus (HeV). The LexA DNA-binding domain (DBD) was fused to the N-terminus of both *Henipaviruses* with an additional construct where the DBD was fused to the C-terminus of NiV M protein (Fig. 4-1A). Fusion of the DBD to both the N- and C- termini of NiV M protein serves to identify any protein-protein interactions that may be sterically hindered

with one particular fusion protein. These bait constructs were tested for self-activation of reporter genes using a differential growth test on selection media and for non-specific activation of the LacZ reporter. All three of the *Henipavirus* M protein baits failed to grow on media selecting for protein-protein interactions and were unable to activate the lacZ reporter as tested by filter β -galactosidase assays (Fig. 4-1B and data not shown). The efficient expression of each *Henipavirus* M protein fusion was also verified via western blot with anti-LexA monoclonal antibody (Fig. 4-1C and data not shown). Taken together these data show that the *Henipavirus* M protein baits were suitable for yeast two-hybrid screening.

Yeast 2-hybrid screening was employed using a yeast 2-hybrid mating strategy. *Henipavirus* M protein bait containing L40 yeast were mated with EG48 containing a cDNA library containing 5.7×10^6 primary clones derived from human lung fibroblasts (HLF). The mating yielded a diploid strain of yeast where transcription of LexA-driven reporters produced β -galactosidase and allowed for the yeast to grow on media lacking uracil, leucine, and histidine. Approximately 3×10^7 clones were screened for each of the *Henipavirus* M protein baits that had the DBD fused to their N-termini while 10 times more clones needed to be screened when the DBD was fused to the C-terminus (Fig. 4-1D). This discrepancy was due to a dramatic difference in screening efficiency. When the DBD was fused to the N-terminus of the *Henipavirus* M protein baits, screening was extremely efficient leading to a high number of β -galactosidase positive colonies. However, when the DBD was fused to the C-terminus of NiV M protein there was a dramatic reduction in the number of β -galactosidase positive clones identified (Fig. 4-1D). This was potentially a result of poor bait protein expression (Fig. 4-1C).

Positive yeast clones identified during screening were isolated and grown on selection media. If colonies were able to grow after isolation, candidates were then tested for activation of the lacZ reporter gene using a colony filter assay testing for β -

galactosidase activity. Candidates testing positive for β -galactosidase activity turned blue indicating a potential *Henipavirus* M-host protein interaction while colonies that remained white were considered false-positives and discarded (data not shown). Following selection using these two reporter systems, yeast plasmid DNA was isolated from the yeast candidates and their cDNA inserts were amplified using PCR. Following amplification, PCR products were sequenced and identified using the GenBank database. In total, more than 200 candidates were identified and broken into groups based on the bait constructs they were identified with (Table 4-1 and 4-2).

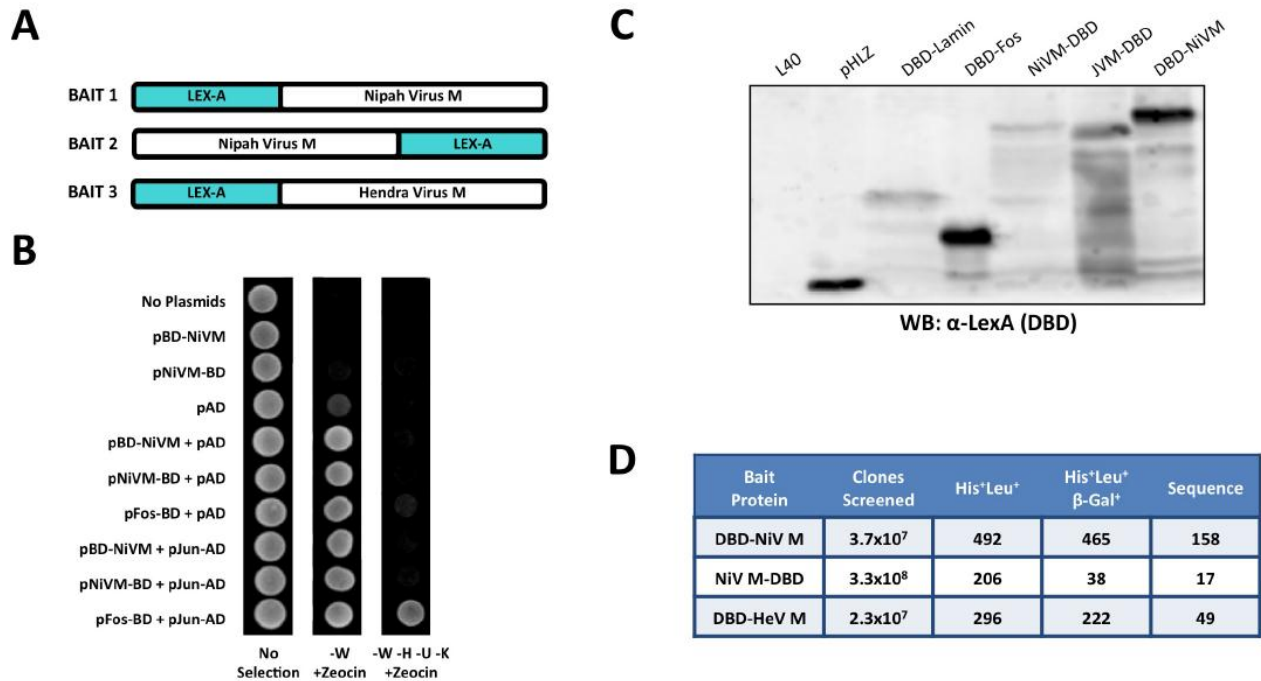


Fig. 4-1 Yeast 2-hybrid screening of *Henipavirus M* proteins

(A) NiV and HeV cDNA was amplified and cloned into pHLZ (Invitrogen) yeast expression vector. The LexA DNA-binding domain was fused to the N-terminus of both *Henipavirus M* proteins and LexA was fused to C-terminus of only NiV M protein. (B) Transformed yeast was patched onto nutrient dropout media lacking uracil, histadine, and lysine to test for self-activation of reporter genes. Bait and prey plasmids were maintained with media containing Zeocin and lacking tryptophan. (C) Yeast extracts were for bait protein expression by immunoblotted with α-Lex A antibody. The NiVM-DBD construct had much lower levels of expression in L40 as compared with other constructs fused at their N-terminus with the DBD. (D) A table summarizing the yeast 2-hybrid screening statistics for each *Henipavirus M* protein bait. The screening of the NiV M-DBD bait protein was much less efficient with far fewer His⁺ Leu⁺ β-Gal⁺ colonies as compared to the other bait proteins screened. Of the baits screened, >200 of the prey plasmid containing clones were identified by sequencing the cDNA insert with vector specific primers.

Table 1: Candidate binding partners for NiV DBD-M

Bait Protein	Prey Protein Identified	Times Isolated
DBD-NiV M	fibroblast growth factor (acidic) intracellular binding protein (FIBP)	57
DBD-NiV M	casein kinase 2, alpha 1 polypeptide (CSNK2A1)	19
DBD-NiV M	B-cell CLL/lymphoma 9-like (BCL9L)	12
DBD-NiV M	<i>midnolin (MIDN) *</i>	11
DBD-NiV M	TSPY-like 2 (TSPYL2)	8
DBD-NiV M	retinoblastoma binding protein 6 (RBBP6)	6
DBD-NiV M	<i>ribosomal protein L10 (RPL10) **</i>	6
DBD-NiV M	ADP-ribosylation-like factor 6 interacting protein 4 (ARL6IP4)	5
DBD-NiV M	kelch repeat and BTB (POZ) domain containing 7 (KBTBD7)	5
DBD-NiV M	clusterin associated protein 1 (CLUAP1)	4
DBD-NiV M	low density lipoprotein-related protein 1 (LRP1)	3
DBD-NiV M	nucleosome assembly protein 1-like 1 (NAP1L1)	2
DBD-NiV M	protein inhibitor of activated STAT, 3 (PIAS3)	2
DBD-NiV M	R3H domain containing 2 (R3HDM2)	2
DBD-NiV M	transgelin (TAGLN)	2
DBD-NiV M	casein kinase 2, alpha prime polypeptide (CSNK2A2)	1
DBD-NiV M	chromodomain helicase DNA binding protein 8 (CHD8)	1
DBD-NiV M	<i>Chromosome 4 genomic contig *</i>	1
DBD-NiV M	<i>coatmer protein complex, subunit epsilon (COPE) *</i>	1
DBD-NiV M	coil-coil domain-containing-like (LOC126075)	1
DBD-NiV M	coiled-coil domain-containing-like (LOC126075)	1
DBD-NiV M	cyclin M2 (CNNM2)	1
DBD-NiV M	death-domain associated protein (DAXX)	1
DBD-NiV M	DnaJ (Hsp40) homolog, subfamily B, member 12 (DNAJB12)	1
DBD-NiV M	MAX interactor 1 (MXI1), var 1	1
DBD-NiV M	nuclear factor kappa light polypeptide gene enhancer in B-cells (NFKBIL1)	1
DBD-NiV M	ornithine decarboxylase antizyme 1 (OAZ1)	1
DBD-NiV M	peroxisome proliferator-activated receptor coactivator-related 1 (PPRC1)	1
DBD-NiV M	thrombospondin 2 (THBS2)	1

* cDNA insert outside translated region of candidate ORF

** Typical 2-Hybrid false positive

Total 158

Table 4-1 Candidate binding partners for DBD-NiV M protein.

The identified candidate NiV M-binding proteins were sorted based on the number of times each clone was isolated. Sequences were obtained following plasmid isolation and PCR amplification of prey library plasmid inserts. The candidates highlighted in bold were selected to confirm interaction with NiV M protein in mammalian cells. Some cDNA sequences identified could not produce the identified protein as indicated with a *. Typical yeast 2-hybrid false positives are indicated with a **.

Table 2: Candidate binding partners for NiV M-DBD and HeV DBD-M

Bait Protein	Prey Protein Identified	Times Isolated
NiV M-DBD	kelch repeat and BTB (POZ) domain containing 7 (KBTBD7)	5
NiV M-DBD	thioredoxin-like 1 (TXNL1)	2
NiV M-DBD	bromodomain and PHD finger containing, 1 (BRPF1)	1
NiV M-DBD	calreticulin (CALR)	1
NiV M-DBD	chromodomain helicase DNA binding protein 8 (CHD8)	1
NiV M-DBD	DnaJ (Hsp40) homolog, subfamily B, member 14 (DNAJB14)	1
NiV M-DBD	fibroblast growth factor (acidic) intracellular binding protein (FIBP)	1
NiV M-DBD	general transcription factor IIF, polypeptide 1, 74kDa (GTF2F1)	1
NiV M-DBD	H3 histone, family 3B (H3.3B) (H3F3B)	1
NiV M-DBD	nucleolar and coiled-body phosphoprotein 1 (NOLC1)	1
NiV M-DBD	peptidylprolyl isomerase B (cyclophilin B) (PPIB)	1
NiV M-DBD	zinc finger, FYVE domain containing 1 (ZFYVE1)	1
Total		17

Bait Protein	Prey Protein Identified	Times Isolated
DBD-HeV M	fibroblast growth factor (acidic) intracellular binding protein (FIBP)	19
DBD-HeV M	B-cell CLL/lymphoma 9-like (BCL9L)	8
DBD-HeV M	peptidylprolyl isomerase B (cyclophilin B) (PPIB)	7
DBD-HeV M	chromodomain helicase DNA binding protein 8 (CHD8)	5
DBD-HeV M	casein kinase 2, alpha 1 polypeptide (CSNK2A1)	3
DBD-HeV M	clusterin associated protein 1 (CLUAP1)	2
DBD-HeV M	<i>ribosomal protein L10 (RPL10) **</i>	2
DBD-HeV M	retinoblastoma binding protein 6 (RBBP6)	1
DBD-HeV M	nuclear factor of kappa light polypeptide in B-cells inhibitor-like 1 (NFKBIL1)	1
DBD-HeV M	Treacher Collins-Franceschetti syndrome 1 (TCOF1)	1
Total		49

* cDNA insert outside translated region of candidate ORF

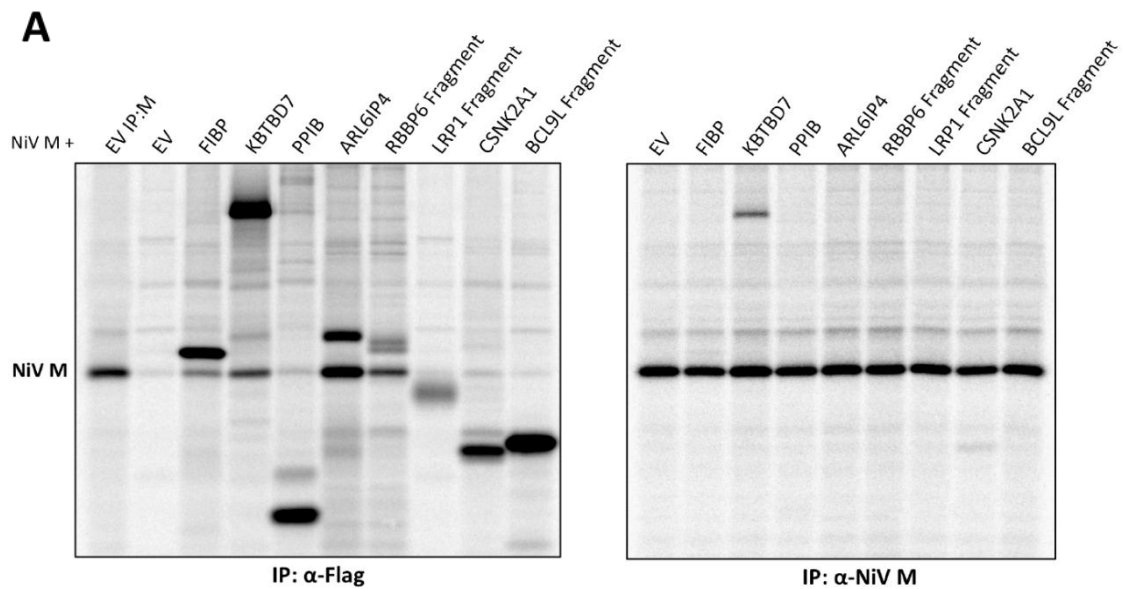
** Typical 2-Hybrid false positive

Table 4-2 Candidate binding partners for NiV M-DBD and DBD-HeV M proteins

The identified candidate NiV M-binding proteins were sorted based on the number of times each clone was isolated. Sequences were obtained following plasmid isolation and PCR amplification of prey library plasmid inserts. The candidates highlighted in bold were selected to confirm interaction with NiV M protein in mammalian cells. Some cDNA sequences identified could not produce the identified protein as indicated with a *. Typical yeast 2-hybrid false positives are indicated with a **.

Confirmation of Protein Interactions in Mammalian Cells

Several putative *Henipavirus* M-interacting host proteins were identified numerous times and also independently confirmed with multiple bait constructs. Preference was given to these identified candidates to secondarily confirm their interaction with NiV M protein. Of the host proteins identified, the following were selected for further study: FIBP, CSNK2A1, KBTBD7, ARL6IP4, PPIB, RBBP6, LRP1, and BCL9L (highlighted in bold Tables 4-1 and 4-2). When possible full-length cDNAs of the candidate NiV M-interacting protein were obtained and cloned into a mammalian expression vector, pCAGGS, in frame with a Flag epitope tag for detection. HEK293T cells transfected to express a Flag-tagged candidate protein and NiV M protein were tested for binding using co-immunoprecipitation assays. Of the eight candidate proteins selected for secondary confirmation of binding, five co-precipitated with NiV M protein in mammalian cells: acidic fibroblast growth factor intracellular-binding protein (FIBP), casein kinase II subunit alpha (CSNK2A1), kelch repeat and BTB (POZ) domain containing protein 7 (KBTBD7), ADP-ribosylation-like factor 6 interacting protein 4 (ARL6IP4), and retinoblastoma binding protein 6 (RBBP6) (Fig. 4-2A). These results have confirmed the binding between NiV M protein and several host proteins with a variety of known cellular functions (Fig. 4-2B).



B

Y2H Candidate	M Protein Binding	Protein Function
ARL6IP4	+++	May act as a splicing inhibitor of HSV-1 pre-mRNA
BCL9L	-	Transcriptional regulator; Role in tumorigenesis
CSNK2A1	+/-	Serine/threonine protein kinase that phosphorylates acidic proteins
FIBP	+	Growth factor that stimulates mitogenesis; Binds aFGF
KBTBD7	++	Likely involved in substrate ubiquitination; Binds CUL3
LRP1	-	Intracellular signaling; Lipid homeostasis
PPIB	-	Cyclosporine-binding protein; Potentially involved in protein folding
RBBP6	++	E3 ubiquitin ligase; Promotes YBX1 ubiquitination; May negatively regulate p53

Fig. 4-2 Confirmation of binding between Y2H candidates and NiV M protein by Co-IP.

(A) NiV M protein was co-expressed with Flag tagged yeast two-hybrid candidate proteins in HEK293T cells to confirm binding of yeast 2-hybrid candidates with NiV M protein. Cells were labeled ^{35}S for 3hrs. before cell lysates were prepared. NiV M protein alone controls were immunoprecipitated with either anti-NiV M antibody or FLAG[®] magnetic affinity resin. Samples co-expressing candidate proteins with NiV M protein were immunoprecipitated with anti-Flag resin or anti-NiV M polyclonal antibody. Immunoprecipitated cell lysates analyzed on 15% SDS-PAGE gel. Gels were exposed on a storage phosphor screen overnight and scanned on a Fuji FLA-7000 laser scanner. (B) Summary of the secondary screening for NiV M-host protein interactions in mammalian cells. The number of '+' marks denotes the relative strength of interaction as judged by the total amount of NiV M protein co-immunoprecipitated with the host protein. A +/- denotes a weak binding as detected using this method. Also included is a brief summary of the known functions of these host proteins.

Impact of overexpressed NiV M protein binding partners on VLP production

The overexpression of full-length host proteins that bind to HIV-1 gag can cause severe defects in virus budding. For example, overexpression of TSG101 causes the formation of aberrant endosomal compartments and severe reductions in HIV-1 particle release (46). To test if the NiV M-interacting host proteins identified here might impact Nipah VLP production similar to the negative effect of TSG101 overexpression on HIV-1 assembly, NiV M-interacting proteins were overexpressed with NiV M protein in HEK293T cells to measure their effects on Nipah VLP release. Following the metabolic labeling of transfected cells, VLPs were collected from culture media and purified on sucrose density gradients and loaded directly on SDS gels. Most of the full-length host proteins did not have any effect on Nipah VLP production when overexpressed (Fig. 4-3). However, there were some effects on Nipah VLP production by peptidyl-prolyl cis-trans isomerase B (PPIB) and ADP-ribosylation-like factor 6 interacting protein 4 (ARL6IP4). Overexpression of PPIB with NiV M protein caused a 3-fold reduction in VLP production, but high levels of cytotoxicity confounded this observation. The only other host protein to decrease Nipah VLPs was ARL6IP6. The overexpression of ARL6IP4 reduced Nipah VLP production by 2-fold and could represent a potentially important factor in particle assembly and budding. The Nipah VLP production efficiency was calculated based on the amount of NiV M protein in the media normalized to the level of NiV M protein in the cell lysates. This result indicates that the overexpression of full-length NiV M-interacting proteins had only minor impacts on Nipah VLP production.

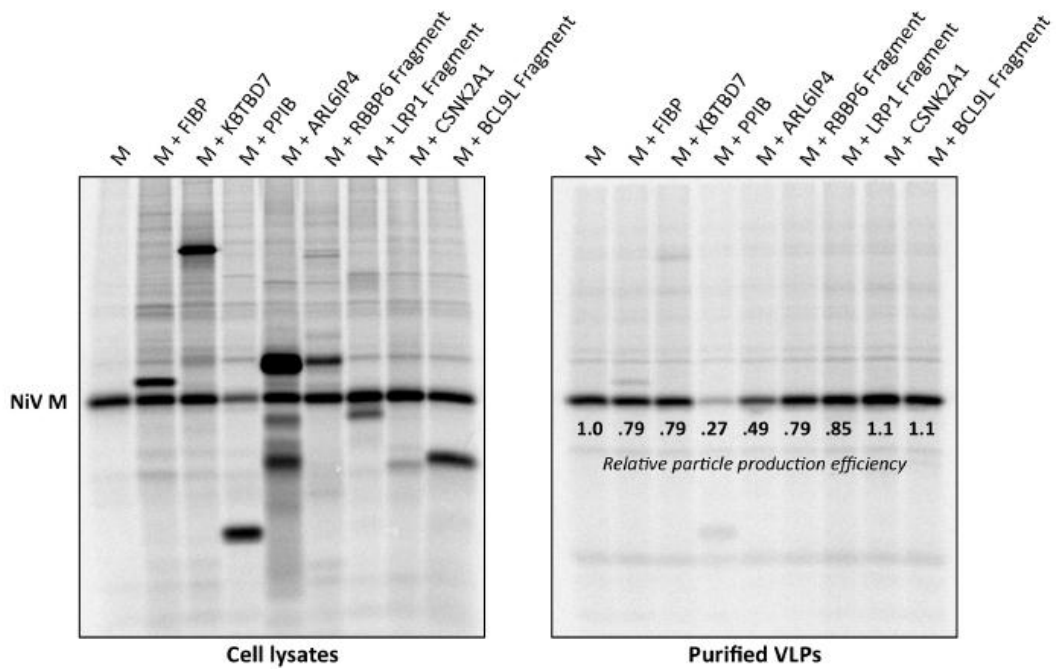


Fig. 4-3 The effects of overexpression of Y2H candidate proteins on protein of Nipah VLPs. HEK293T were transfected to expressing NiV M protein together with normalized plasmid amounts of full-length yeast two-hybrid candidates. 24h p.t. cells were labeled with ^{35}S for 18h before Nipah VLPs were then purified. Cell lysates were immunoprecipitated with anti-NiV M antibody and FLAG[®] magnetic affinity resin. Samples were resolved on 15% SDS-PAGE and detected using a storage phosphor imaging system. Particle production efficiency was calculated as the amount of NiV M protein in purified particles divided by the amount in the cell lysate fraction and normalized to the sample with NiV M protein by itself.

Mapping the NiV M protein binding regions in KBTBD7

The KBTBD7 protein is an evolutionarily conserved 685 amino acid long protein that is expressed in numerous cell types (64). Although relatively little is known about KBTBD7, it is member of a family of other BTB domain containing proteins and has been implicated to play a role in the human autophagy system and in activation of mitogen-activated protein kinase (MAPK) pathways (6, 64). Other functions attributed to this family of proteins are highly variable including: transcriptional regulation, cytoskeleton regulation, gating of ion channels and protein degradation via the ubiquitin proteasome pathway (74, 77, 82, 103, 106). The N-terminal region of KBTBD7 contains a BTB-domain and a BACK domain while the C-terminal region contains kelch repeats for binding substrate proteins. The majority KBTBD7 prey sequences identified during yeast 2-hybrid screening corresponded to the full-length protein; however, a small number of C-terminal fragments were identified that corresponded to the amino acid residues 578-685 of KBTBD7 (Fig. 4-4B).

In an effort to define the minimal region required for binding of KBTBD7 with NiV M protein, truncations were made to KBTBD7 based on the binding fragment identified during yeast 2-hybrid screening. To confirm if this region was sufficient for binding to NiV M protein, HEK293T cells were transfected to express KBTBD7 1-577 and KBTBD7 578-685 with NiV M protein were radiolabeled and immunoprecipitated. Only the immunoprecipitation of the KBTBD7 578-685 fragment was able to co-precipitate NiV M protein similar to full-length KBTBD7 (Fig. 4-4A). To further define the region required for KBTBD7's binding with NiV M protein additional C-terminal truncations of KBTBD7 were created (Fig. 4-4). Co-immunoprecipitation experiments with these truncation mutants revealed that truncation of KBTBD7 beyond amino acid 665 was not compatible with binding to NiV M protein (Summarized in Fig. 4-4B). In an attempt to identify the

shortest KBTBD7 region sufficient for binding with NiV M protein, several small peptides were also constructed based on these findings. However, only the original peptide identified, KBTBD7 578-685, was sufficient to interact with NiV M protein (data not shown). Together these results have identified a small KBTBD7 fragment sufficient for interaction with NiV M protein and have defined several amino acids that are required for KBTBD7-NiV M protein binding.

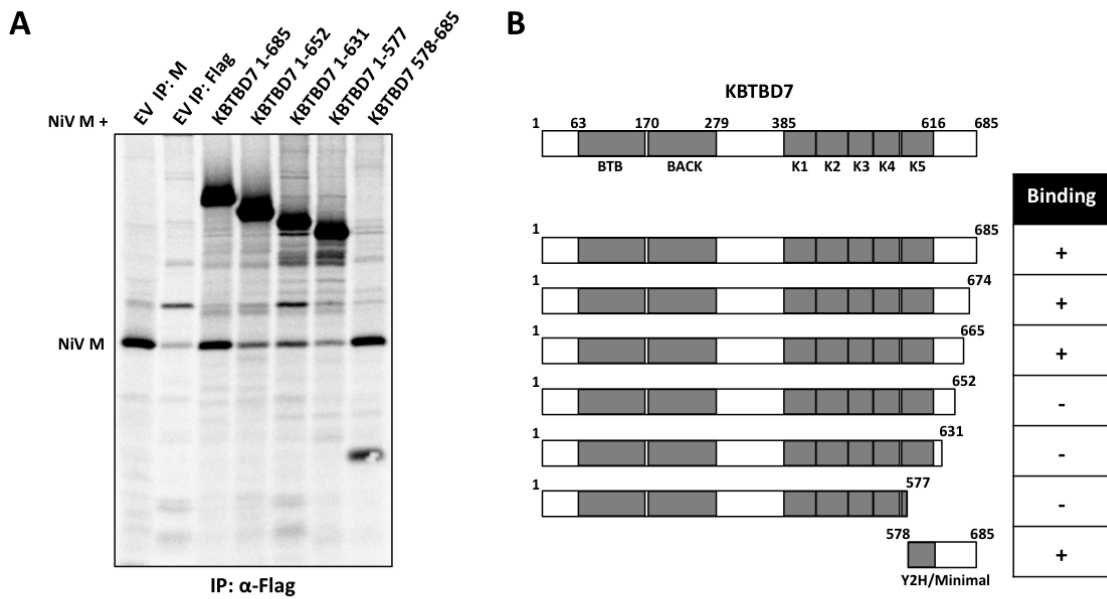


Fig. 4-4 Identification of an KBTBD7 minimal binding fragment

(A) HEK293T cells transfected to express KBTBD7 or derivatives with NiV M protein were tested for co-immunoprecipitation using the method described above. **(B)** Schematic illustration of KBTBD7 C-terminal truncations and the yeast 2-hybrid fragment tested for binding to NiV M protein. A table also summarizes which KBTBD7 derivative is positive or negative for NiV M protein binding.

Mapping the NiV M protein binding regions in ARL6IP4

To further extend these mapping studies, ARL6IP4 was selected for further study. ARL6IP4, also known as SR-25 belongs to a superfamily of proteins that contain repetitive serine-arginine sequences known as SR domains. SR domain-containing proteins are involved in regulation of mammalian mRNA splicing and this activity is likely regulated through the phosphorylation of the SR domains (Reviewed in 95). Additionally, SR domain-containing proteins can inhibit virus replication. For example, overexpression of the SR-15 protein is able to inhibit herpes simplex virus 1 (HSV1) replication through suppression of viral pre-RNA splicing in the nucleus (88). Although it is unclear how ARL6IP4 may be involved in *Henipavirus* replication a further understanding of its interaction with M protein may result in the development of new tools to investigating the functional relevance of this interaction.

To this end, the minimal region required for binding between NiV M and ARL6IP4 was mapped. A series of plasmids were constructed that encoded N- and C- terminal truncations of ARL6IP4 in an effort to identify the critical regions required for NiV M protein binding. One of these peptides, ARL6IP4 181 to 271, corresponded to the cDNA clone that was isolated during yeast 2-hybrid screening. To identify the region important for binding to NiV M protein, HEK293T cells were transfected to express several ARL6IP4 fragments with NiV M protein. Only immunoprecipitation of ARL6IP4 fragments that contained amino acids 181 to 271 were able to co-precipitate NiV M protein (Fig. 4-5). However, the binding status of two fragments that were presumed to bind could not be determined due to their close molecular weight with NiV M protein. These mapping studies have resulted in the identification of a short stretch of serine and basic residues in the center of ARL6IP4 to be essential for binding to NiV M protein.

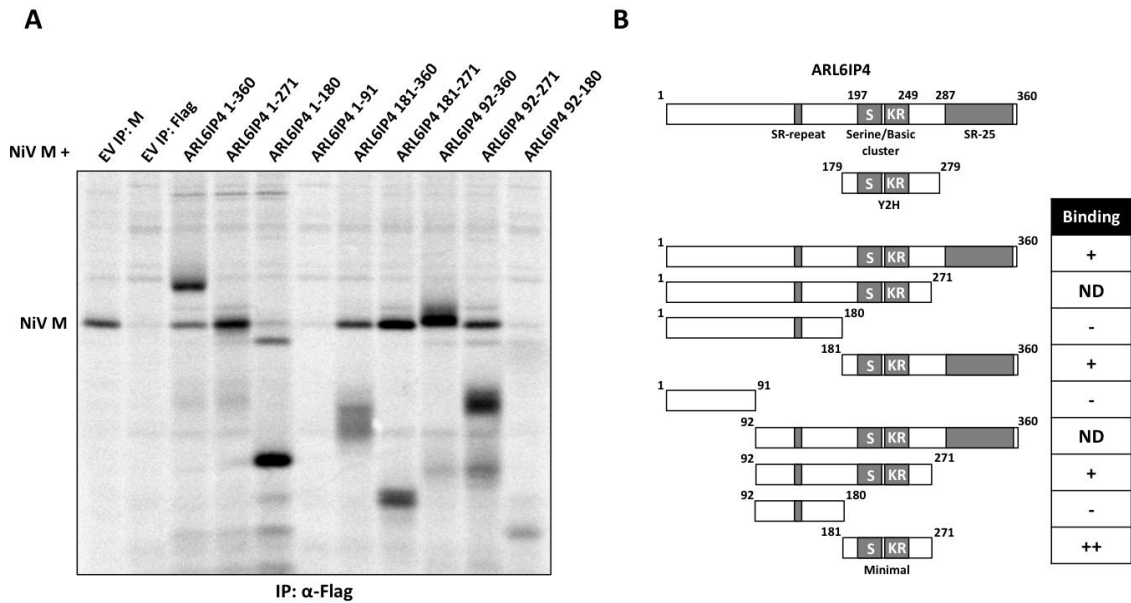


Fig. 4-5 Identification of an ARL6IP4 minimal binding fragment

(A) HEK293T cells transfected to express ARL6IP4 or derivatives with NiV M protein were tested for co-immunoprecipitation using the method described above. **(B)** Schematic illustration of ARL6IP4 fragments tested for binding with NiV M protein. The serine/basic region represents a continuous cluster of 25 serine residues labeled “S” and a region mostly comprised of lysine and arginine residues labeled “KR”. A table also summarizes which ARL6IP4 derivative is positive or negative for NiV M protein binding. ND indicates that the binding was not determined due to close migration of the ARL6IP4 fragment and NiV M protein.

Inhibition of Nipah VLP production using small peptide inhibitors

Following a previous yeast 2-hybrid screen of PIV5 M protein, our laboratory identified a minimal M-binding peptide, m-AMOTL1, that was able to significantly reduce PIV5 VLP production as described in 4.1 (119). Using a similar approach, minimal binding peptides were developed for KBTBD7 and ARL6IP4 proteins and tested for their ability to inhibit Nipah VLP production. In addition to the KBTBD7 and ARL6IP4 peptides, another peptide derived from the adaptor protein complex 3 beta 1 subunit (AP3B1), was identified using a co-affinity purification of HeV M protein followed by mass spectrometry identification of co-purified peptides. Mapping studies of the NiV M-AP3B1 binding region identified a minimal binding peptide, AP3B1 (643-705), which bound with NiV M protein. In addition to binding with NiV M protein, AP3B1 (643-705) also inhibited Nipah VLP production (In preparation Sun, et al., unpublished). Hence, in these experiments, AP3B1 (643-705) serves as a control peptide known to inhibit Nipah VLP production.

To test if the minimal binding peptides derived from KBTBD7 and ARL6IP4 could inhibit Nipah VLP production, HEK293T cells were transfected to express NiV M-interacting peptides with two different amounts of NiV M protein plasmid DNA. Varied amounts of NiV M protein expression were used to test the relative ability of each peptide to inhibit different levels of Nipah VLP production. Nipah VLPs were collected and purified from HEK293T cells transfected to express NiV M protein with each of the small M-binding peptides. The overexpression of KBTBD7 (578-685) and AP3B1 (643-705) both reduced Nipah VLP production by ~3-fold as compared to the empty vector control sample when NiV M protein was expressed at lower levels (Fig. 4-6). However, when NiV M protein expression was increased this reduction in VLP production was minimal. This suggests that when expressed in high enough quantities, NiV M protein

can overcome the inhibitory effect of these polypeptides. In contrast, when ARL6IP4 (181-271) was overexpressed, there was a more moderate reduction in Nipah VLP production of 2-fold, and this reduction was relatively unaffected by increasing NiV M protein expression (Fig. 4-6). Together, these results have identified three NiV M-binding peptides that potentially block Nipah VLP production when overexpressed in mammalian cells.

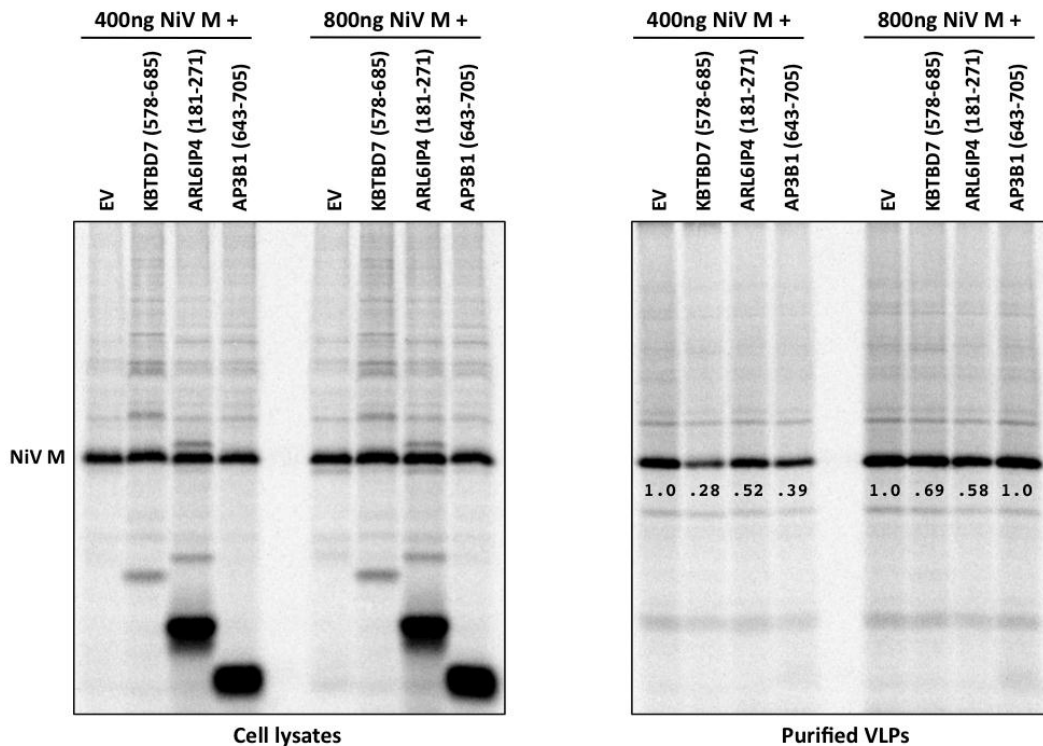


Fig. 4-6 Disruption of Nipah VLPs using small peptide binding fragments.

HEK293T cells were transfected with 0.4 or 0.8 μ g of NiV M plasmid DNA and 1.0 μ g plasmid DNA encoding either KBTBD7, ARL6IP4 or AP3B1 M-binding fragments. VLPs were produced and purified as described above. Particle production efficiency was calculated as the amount of NiV M protein in purified particles divided by the amount in the cell lysate fraction and normalized to the sample containing NiV M protein by itself.

Co-affinity purification of *Henipavirus* M following RNase A treatment

In an effort to gain a more comprehensive view of the host factors important for the function *Henipavirus* M proteins, a co-affinity purification of Strep II/His tagged HeV M (SH-HeV M) protein was performed to identify co-purified polypeptides. A previous co-affinity purification of SH-HeV M protein in our laboratory identified many host factors including a member of the AP-3 complex, AP3B1. The interaction of AP3B1 with NiV M protein was extensively characterized and found to be an important factor for virus assembly and budding (In preparation Sun, et al., unpublished). However, 80 percent of the total peptides identified in this co-affinity purification were potentially non-specific RNA-binding host proteins. The majority of these RNA-binding proteins identified were RNA helicases, splicing factors, and ribosomal proteins. This high background of RNA-binding proteins made identification of relevant interactions more difficult and may have prevented identification of other important host factors.

To address this concern, HEK293T cells transfected to express SH-HeV M protein were lysed in low detergent buffer containing physiological levels of salt to maintain protein-protein interactions. The RNA in these cell extracts was then degraded with RNase A at room temperature before FPLC co-affinity purification of SH-HeV M protein associated protein complexes. This RNase A treatment did not result in any significant protein degradation as compared to a control kept at 0°C for the same period of time (data not shown). The purified SH-HeV M protein was then resolved on SDS gels and visualized using coomassie brilliant blue staining. Purification of SH-HeV M protein using this method was able to drastically reduce the complexity of the proteins co-purified with HeV M protein (Fig. 4-7A). This was particularly evident in the lower molecular weight bands; these bands were previously identified to be composed of mainly ribosomal proteins. Of the proteins that were co-purified with SH-HeV M protein,

eight polypeptide bands were excised to be identified using mass spectrometry (Fig. 4-7B). The excised bands migrated at the following molecular weights: #1 ~210 kDa; #2 ~130 kDa; #3 ~120 kDa; #4 ~114 kDa; #5 ~112 kDa; #6 ~110 kDa; #7 ~70 kDa; #8 ~35 kDa.

The polypeptide composition of the eight bands selected was identified using mass spectrometry. Each of the excised bands was subjected to in-gel trypsin digestion and the resulting tryptic fragments were identified using liquid chromatography tandem mass spectrometry. This analysis was performed by the Taplin Mass Spectrometry Facility at Harvard University. Analysis of the identified peptides found that there was a 2.5-fold reduction in the number of RNA-binding proteins identified as compared to a previous co-affinity purification of SH-HeV M protein (Summarized in Fig. 4-8). In addition to removing many of the RNA-binding proteins, many new proteins were identified in the absence of RNA including: ACACA, BRD4, EIF3A, EIF3B, EIF3C, VPRBP, HTATSF1, KDM1, and HSPA8. Several of the identified proteins (AP3B1, AP3D1, TCOF1, NCL, SUPT16H, DDB1, PCCA, and PABPC1) were also identified in an earlier screen where RNase A pretreatment was not used (In preparation Sun, et al., unpublished). Taken together this procedure resulted in a dramatic reduction in co-purified RNA-binding host proteins while preserving the interactions with many previously identified host proteins. Additionally, several previously unidentified host proteins were identified when most of the RNA-binding proteins were removed.

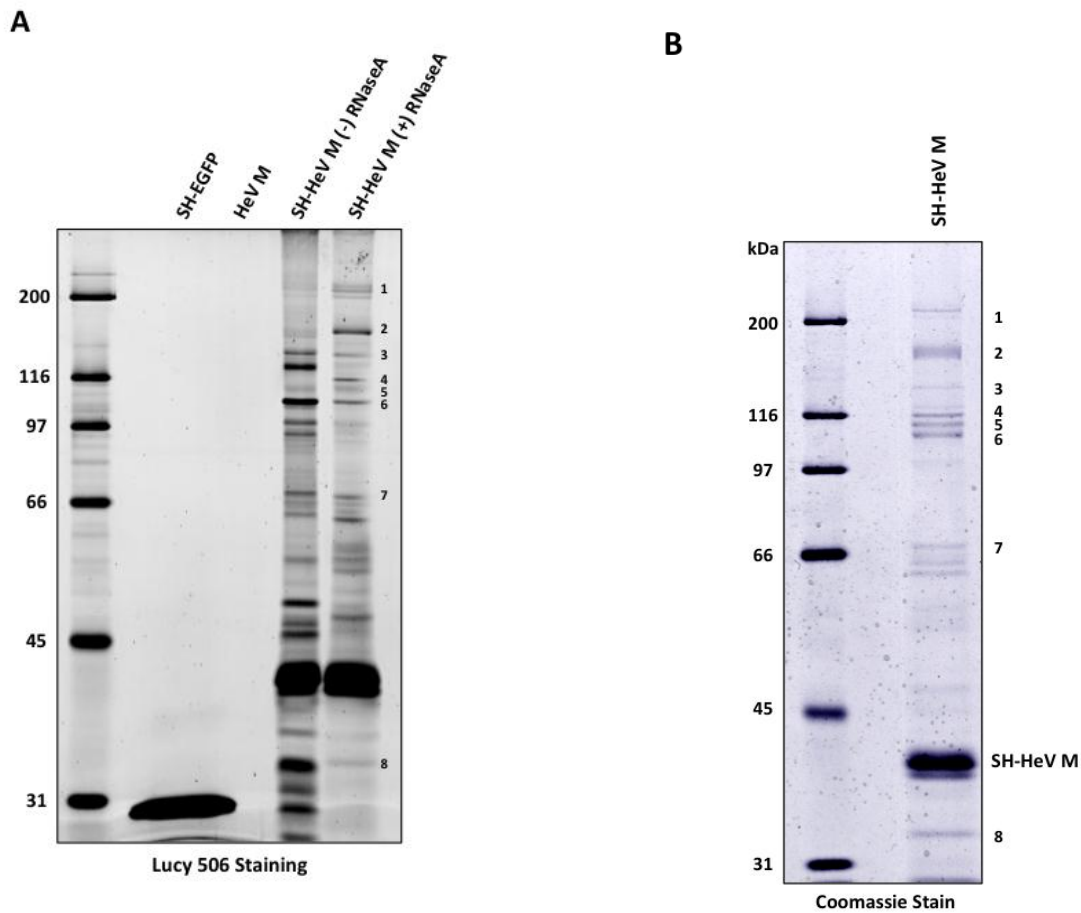


Fig. 4-7 Co-affinity purification of NiV M protein following RNase A treatment.

(A) HEK293T cells were transfected with 3 μ g of either SH-EGFP or SH-HeV M plasmid. 24.h p.t. cells were lysed in FPLC binding buffer A containing 0.5% NP-40 and incubated with 200 μ g/ml of RNase A for 30 min. at 20°C were indicated. Lysates were then filtered before being purified on the FPLC. The elution fractions were concentrated and a small portion of the purified protein was resolved on a 10% SDS-PAGE gel and immunoblotted with α -NiV M antibody to determine the relative efficiency of the purification with and without RNase A treatment. Normalized levels of purified SH-HeV M protein were loaded onto 10% gels and polypeptides were visualized using Lucy 506 protein staining. **(B)** SH-HeV M protein was purified as described above using a scaled up procedure to select bands for mass spec identification. Proteins were visualized using coomassie brilliant blue protein staining.

Band ID	Unique Peptides	Coverage	Protein Name
1 ~210kd	125	45%	Acetyl-CoA carboxylase 1 (ACACA)
	34	16%	Small nuclear RNP helicase (SNRNP200)
	29	24%	Treacher Colling-Franceschetti syndrome 1 (TCOF1)
	25	16%	Bromodomain-containing protein 4 (BRD4)
	25	12%	Pre-mRNA splicing factor (PRPF8)
2 ~130kd	111	52%	Eukaryotic translation initiation factor (EIF3A)
	31	25%	Treacher Colling-Franceschetti syndrome 1 (TCOF1)
	27	24%	AP-3 complex subunit delta-1 (AP3D1)
	16	17%	HIV-1 Vpr binding protein (VPRBP)
	15	9%	Acetyl-CoA carboxylase 1 (ACACA)
3 ~120kd	52	39%	SUPT16H FACT complex (SUPT16H)
	34	29%	Treacher Colling-Franceschetti syndrome 1 (TCOF1)
	18	19%	Ubiquitin carboxyl-terminal hydrolase 7 (USP7)
	15	27%	HIV Tat-specific factor 1 (HTATSF1)
	12	12%	AP-3 complex subunit beta-1 (AP3B1)
4 ~114kd	61	51%	Eukaryotic translation initiation factor (EIF3B)
	45	29%	Eukaryotic translation initiation factor (EIF3A)
	44	41%	U5 small nuclear RNP component (EFTUD2)
	33	26%	DNA damage-binding protein 1 (DDB1)
	32	28%	Treacher Colling-Franceschetti syndrome 1 (TCOF1)
5 ~112kd	52	43%	Eukaryotic translation initiation factor (EIF3CL)
	43	28%	Eukaryotic translation initiation factor (EIF3A)
	28	34%	Eukaryotic translation initiation factor (EIF3B)
	26	20%	Treacher Colling-Franceschetti syndrome 1 (TCOF1)
	24	36%	Nucleolin (NCL)
6 ~110kd	52	33%	Eukaryotic translation initiation factor (EIF3A)
	37	35%	Eukaryotic translation initiation factor (EIF3CL)
	35	50%	Nucleolin (NCL)
	35	31%	WASH complex subunit strumpellin (KIAA0196)
	31	35%	Lysine-specific histone demethylase 1 (KDM1)
7 ~70kd	52	51%	Propionyl-CoA carboxylase alpha chain (PCCA)
	46	58%	Heat shock cognate 71 (HSPA8)
	34	39%	Trifunctional enzyme subunit alpha (HADHA)
	28	22%	Treacher Colling-Franceschetti syndrome 1 (TCOF1)
	24	36%	Polyadenylate-binding protein (PABPC1)
8 ~35kd	26	58%	Mitochondrial ribosomal protein (MRPL45)
	22	48%	Acidic (leucine-rich) nuclear phosphoprotein 32 (ANP32B)
	22	59%	Ribosomal protein (PS3A)
	20	70%	Ribosomal protein (RPS3)
	11	39%	Mitochondrial ribosomal protein (MRPL15)

Table 4-3 Identified HeV M protein candidate binding proteins by co-affinity purification following RNase A treatment.

A summary of the top five identified proteins identified by mass spec for each polypeptide band selected for identification. Identified proteins are sorted based on the total number of unique peptides identified. The coverage refers to the percent overlap of the combined peptides identified with the known protein amino acid sequence.

Confirmation of candidate host protein binding with NiV M protein

Numerous host proteins identified following co-affinity purification of SH-HeV M protein were selected to confirm their binding with M protein. This selection was based on the number of unique peptides identified and the commercial availability of full-length cDNAs. The following candidate *Henipavirus* M-interacting proteins were selected: EIF3A, EIF3B, EIF3C, VPRBP, HTATSF1, PARP1, NCL, KIAA0196, CLTC, PABPC1, and HSPA8. Each these newly-identified *Henipavirus* M-interacting candidate proteins were amplified from a full-length cDNA clone and inserted into a mammalian expression vector, pCAGGS, in frame with either a Myc or Flag epitope tag. They were then tested for their ability to co-precipitate with NiV M protein using co-immunoprecipitation assays. HEK293T cells transfected to express an individual host protein with NiV M protein were lysed in buffer containing low levels of detergent and physiological levels of salt to maintain protein-protein interactions. Immunoprecipitation of EIF3B, EIF3C, PARP1, NCL, or HSPA8 resulted in co-precipitation of NiV M protein with varied efficiencies. These results have identified several new host proteins confirmed to bind with NiV M protein in mammalian cells.

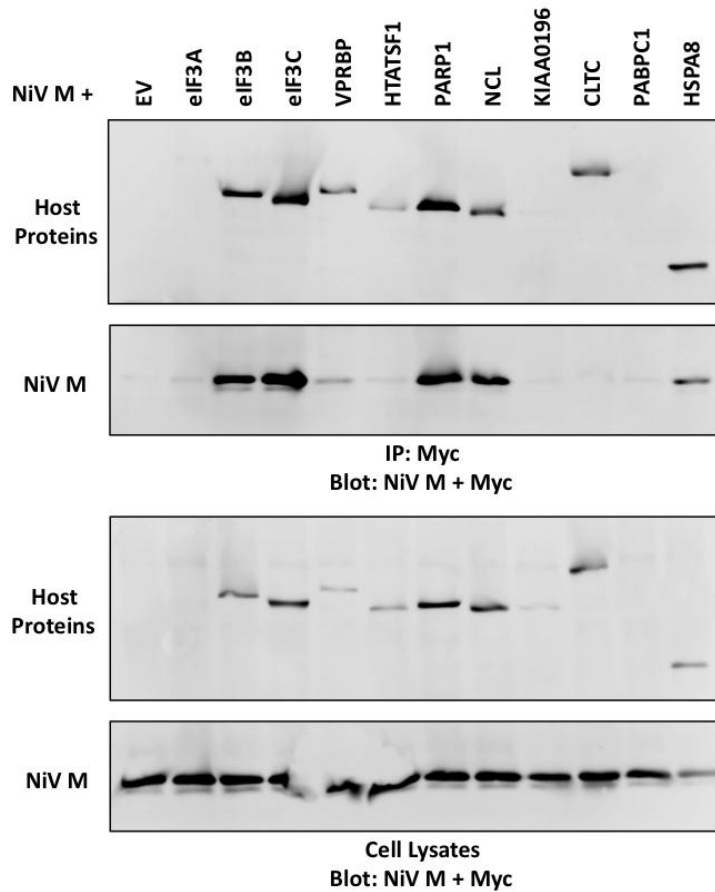


Fig. 4-9 Confirmation of binding of co-affinity purified candidates and NiV M protein. HEK293T cells expressing the indicated host proteins were tested for binding with NiV M protein using co-immunoprecipitation technique as described above. Host proteins co-expressed with NiV M protein were immunoprecipitated with α -Myc (9E10) monoclonal antibody. Cell lysates and immunoprecipitated proteins were detected by immunoblotting with α -NiV M polyclonal antibody and α -Myc (9E10) monoclonal antibody.

Identification of known host protein complexes

Of the confirmed *Henipavirus* M-interacting proteins described here and previously in our laboratory, several are known to interact in a complex with each other. Additionally, some of these complexes have been implicated as important factors for the replication of other viruses. The complexes identified can be grouped as follows based on their known cellular functions: transcriptional control, protein synthesis, post-translational modification, and protein targeting/sorting (Table 4-3). Also summarized in table 4-3 is the known or predicted protein function of each member of the protein complex with the confirmed *Henipavirus* M-interacting proteins being highlighted in bold.

Transcriptional Control

Multiple protein complexes were identified that regulate cellular transcription. The first complex, tat cofactor-containing RNA Pol II complex, is involved in catalyzing DNA transcription (Table 4-3). Two of the host factors within this complex were identified during co-affinity purification screening, nucleolin (NCL) and HTATSF1. In co-immunoprecipitation experiments only NCL bound with NiV M protein (Fig. 4-9). Typically, NCL functions as a pre-RNA transcription factor along with aiding in ribosome assembly. This cellular complex was implicated as an important factor for HIV-1 tat mediated enhancement of viral transcription (114).

The second transcriptional regulatory complex identified was the REST-Co-REST complex (Table 4-3). The REST-Co-REST complex is involved in transcriptional repression and cell type differentiation. Both RCOR1 and SIN3A were identified during the co-affinity purification of HeV M protein. However, it remains to be tested if these proteins can interact with NiV M protein. The REST-Co-REST complex of proteins plays an important role in the establishment of infection and the establishment of the latent state of herpes simplex virus (HSV) (Reviewed in 130).

A third very large complex of proteins was identified that can all be grouped into the Nop56p-associated preRNA complex (Table 4-3). This large cellular complex is involved in the early stages of ribosome biogenesis and transcriptional control (Characterized in (58)). The host proteins that co-purified with SH-HeV M protein include ACTA2, NCL, ILF2/3, NOLC1, NPM1, SLC25A5, and TCOF1. Two members of this complex, NCL and TCOF1, were confirmed to bind with NiV M protein using co-immunoprecipitation assays (Fig. 4-9 and data not shown). The subunit TCOF1 plays a role in ribosome biogenesis similar to NCL; however, it also is important for the cellular trafficking of proteins between the nucleus and cytoplasm.

Protein synthesis

The co-affinity purification of SH-HeV M protein also resulted in the identification of several eukaryotic initiation factor 3 (EIF3) subunits from the large 800kDa complex, which includes EIF3A, EIF3B, EIF3C, EIF3D, EIF3I and EIF3L (Table 4-3). The function of the EIF3 complex is to stimulate recruitment of initiator tRNA and mRNA to the ribosome for initiation of cellular translation. These subunits are present in distinct stable modules, which are linked together through protein-protein interaction with each other (175). The NiV M protein was found here to interact with both the EIF3B and EIF3C subunits using a co-immunoprecipitation assay (Fig. 4-9). The EIF3 complex has been previously reported to play an important role in several virus systems. For example, subunits of the EIF3 complex are recruited by rabies virus (RV), hepatitis C virus (HCV), and HIV-1 to either inhibit cellular translation, to facilitate viral protein synthesis, or to regulate viral mRNA processing, respectively (79, 140, 155).

Post-translational modification

Two complexes involved in post-translational modification were also identified (Table 4-3). The first was an E3 ubiquitin ligase complex first identified by yeast 2-hybrid

screening then later confirmed in a co-affinity purification (Table 4-1, 4-2 and data not shown). E3 ubiquitin ligase complexes are a large group of proteins best known for their role regulating protein turnover. This is accomplished through the covalent attachment of several small ubiquitin molecules to substrate proteins marking them for proteasome-dependent degradation. The particular E3 ubiquitin ligase components identified here were KBTBD7 and CUL3. Co-immunoprecipitation analysis of these two factors identified that KBTBD7 could interact with both NiV M and CUL3 proteins (Appendix C). Additional testing also found that CUL3 is able to co-precipitate with NiV M protein only when in the presence of KBTBD7 (Appendix C).

Yeast 2-hybrid screening also identified another complex required for phosphorylation, the casein kinase II complex (Table 4-3). This cellular complex serves in a wide variety of functions including cell cycle control and DNA repair. The two subunits of this complex were identified as CSNK2A1 and CSNK2A2. The co-immunoprecipitation data collected between CSNK2A1 and NiV M protein suggested there might be an interaction occurring between the casein kinase II complex and NiV M although it was difficult to confirm (Fig. 4-2). The phosphorylation of viral matrix proteins has been previously shown to play a key role in regulating virus assembly and budding. For example, disruption of Ebola VP40 phosphorylation reduces viral replication by four orders of magnitude (40). Additionally, preventing the phosphorylation of PIV5 M protein renders M protein unable to bind with the cellular protein 14-3-3, which normally functions as a negative regulator of PIV5 particle production (121).

Protein targeting and sorting

The co-affinity purification screens also identified two protein complexes required for the targeting and sorting of proteins (Table 4-3). The first was the profilin1 complex where three subunits were identified: CLTC, HSPA8, and TUBB2B. This cellular

complex plays a role in regulating actin dynamics in response to extracellular stimuli.

NiV M protein was confirmed to bind with the HSPA8 by co-immunoprecipitation (Fig. 4-9). Although the profilin1 complex has not been directly associated with virus budding, regulation of actin dynamics has been linked with retroviral particle production (45).

The adaptor protein 3 (AP3) complex subunits AP3B1 and AP3D1 were also identified following co-affinity purification of SH-HeV M protein (Table 4-3). Typically, the AP3 complex functions as a key mediator of intracellular vesicle transport from the *trans*-Golgi network. The AP3D1 subunit has been shown to interact with HIV-1 gag protein and disruption of this interaction impairs gag protein's function in virus assembly and budding (33). Although AP3D1 was co-purified with SH-HeV M protein, there was no detectable interaction between AP3D1 and M protein using a co-immunoprecipitation assay (data not shown). Instead, the AP3B1 subunit was found to interact with *Henipavirus* M proteins and depletion of endogenous levels of AP3B1 reduces Nipah VLP production (In preparation Sun, et al., unpublished). These data suggest a role for the AP3 complex in the trafficking of *Henipavirus* M protein.

Complex	Identified Protein	Protein Function	Protein-Complex Function
REST-Co-REST complex	RCOR1	Transcriptional repression of neuron-specific genes	Transcriptional control
	SIN3A	Transcriptional repression of MYC responsive genes	
Tat cofactor-containing RNA Pol II complex	NCL**	pre-rRNA transcription and ribosome assembly	
	HTATSF1*	Transcription elongation; co-factor for HIV tat-enhanced transcription	
Nop56p-associated preRNA complex	ACTA2	Cell mobility, structure and integrity	
	NCL**	pre-rRNA transcription and ribosome assembly	
	ILF2/3*	NFAT subunits; transcription factor heterodimer	
	NOLC1	Transcription catalyzed by RNA Pol I; GTPase and ATPase activity	
	NPM1	Protein chaperone; ribosome biogenesis; cell proliferation	
	SLC25A5	Translocation of ADP from mitochondria to cytoplasm	
	TCOF1**	Nuclear cytoplasmic transport; ribosome biogenesis	
EIF3 complex; active core	EIF3A*	Ribosomal mRNA recruitment; AUG scanning	Protein synthesis
	EIF3B**	Ribosomal mRNA recruitment; AUG scanning; central scaffolding subunit	
	EIF3C**	Ribosomal mRNA recruitment; AUG scanning; associates other subunits	
	EIF3I	Ribosomal mRNA recruitment; AUG scanning	
Ubiquitin E3 ligase	KBTBD7**	Likely involved in substrate ubiquitination; binds CUL3	Post-translational modification
	CUL3*	E3 Ubiquitin ligase; mediates substrate ubiquitination and protein degradation	
Casein kinase II complex	CSNK2A1**	Serine/threonine protein kinase that phosphorylates acidic proteins	Protein targeting and sorting
	CSNK2A2	Catalytic subunit that phosphorylates acidic proteins	
Profilin1 complex	CLTC	Major component of clathrin coated pits/vesicles; intracellular trafficking	Protein targeting and sorting
	HSPA8**	Protein chaperone; ATPase that disassembles clathrin coated pits/vesicles	
	TUBB	Major component of microtubules	
AP3 adaptor complex	AP3B1**	Cargo sorting in the TGN/endosomes; recruitment of clathrin to membranes	Protein targeting and sorting
	AP3D1*	Cargo sorting in the TGN/endosomes; budding of vesicles from Golgi	
	AP3M1*	Cargo sorting in the TGN/endosomes; budding of vesicles from Golgi	
	AP3S1	Cargo sorting in the TGN/endosomes; budding of vesicles from Golgi	

**Identified protein binds with NiV M protein *Identified protein does not bind with NiV M protein

Table 4-4 Comprehensive analysis of identified host protein complexes

Host proteins identified during yeast 2-hybrid and co-affinity purification screened were pooled and assessed for their presence in known cellular complexes that are grouped by function: transcriptional control, protein synthesis, post-translational modification, protein targeting and sorting. The binding status for each member of the complex identified is indicated: binds with NiV M protein (**), does not bind with NiV M protein (*), or no mark indicates the binding was not tested.

Serine-rich acidic host factors bind with NiV M protein

Numerous host proteins were confirmed to bind with NiV M protein using co-immunoprecipitation experiments (Listed in 4-10). Of these proteins, minimal binding peptides were independently identified for AP3B1, KBTBD7, and ARL6IP4 (Fig. 4-4, 4-5, and In preparation Sun, et al., unpublished). Interestingly, both of the minimal binding peptides for AP3B1 and KBTBD7 contained similar serine-rich acidic regions. Following this observation, all of the confirmed NiV M-interacting proteins were examined for the presence of serine-rich acidic sequences. Surprisingly, more than half of all NiV M-interacting proteins contained a similar serine-rich acidic region, these included AP3B1, ARL6IP4, EIF3C, KBTBD7, NCL, RBBP6, and TCOF1 (Fig 4-10A). This finding suggests that a similar binding interface may be used by NiV M protein to form electrostatic interactions with a variety of different host proteins.

Mutational analysis of the AP3B1 and KBTBD7 minimal NiV M-binding peptides identified a DSES amino acid sequence that was required for binding to NiV M protein (Fig. 4-4 and In preparation Sun, et al., unpublished). An amino acid sequence alignment of the serine-rich acidic NiV M-interacting proteins identified that five of the seven encoded a similar DSES sequence (Fig. 4-10B). The DSES sequence within these host proteins is potentially required for their interaction with NiV M protein. However, a large homogeneous negatively-charged region also seems to allow for binding to NiV M protein. The two proteins that lack the DSES sequence, ARL6IP4 and NCL, both contain striking stretches of more than 25 consecutive acidic or likely phosphorylated serine residues. Additionally, the minimal NiV-M binding fragment that was identified for ARL6IP4 contained this serine-rich region. Taken together, these results suggest that negatively charged regions may represent an essential characteristic of many NiV M-interacting host proteins.

A

M-interacting Host Protein	Serine-rich Acidic Region	DSES Motif
AP3B1	✓	✓
ARL6IP4	✓	✗
CSNK2A1	✗	✗
eIF3B	✗	✗
eIF3C	✓	✓
FIBP	✗	✗
HSPA8	✗	✗
KBTBD7	✓	✓
NCL	✓	✗
PARP1	✗	✗
RBBP6	✓	✓
TCOF1	✓	✓

B

Alignment of putative serine-rich acidic NiV M-binding regions

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AP3B1 681 EEEDSSDSSS DSESES GSESGEQGES 707
eIF3C 1 *MSRFFPTGS DSESES SLSGEELVTK 16
KBTBD7 643 EWDLDFSEL DSESGS SSSFQDEEVW 668
RBBP6 1626 AAFEPDYNES DSESNV SVKEEESGN 1660
TCOF1 534 PSAQVGKWE DSESSS EESDSSDGE 559

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Fig. 4-10 Serine-rich acidic host proteins interact with NiV M protein.

(A) A schematic summarization of all confirmed NiV M-interacting proteins and if they contain serine-rich acidic regions or DSES motifs. (B) Sequence alignment of the AP3B1, EIF3C, KBTBD7, RBBP6, and TCOF1 serine-rich acidic regions containing DSES motifs. Additional serine and acidic residues also flank these motifs.

4.3 Discussion

This study focuses on the identification of *Henipavirus* M-interacting host proteins. Currently, the host factors required for *Henipavirus* assembly and budding are unknown. Investigation into the requirements for *Henipavirus* particle production has demonstrated that the NiV M protein does not require ESCRT machinery, which is required by many enveloped viruses for particle assembly and budding (118). To identify host factors that are important for *Henipavirus* M proteins function, both yeast 2-hybrid and co-affinity purification screens were performed. These screens resulted in the identification of numerous potential *Henipavirus* M-interacting host proteins. Of these, AP3B1, ARL6IP4, CSNK2A1, EIF3B, EIF3C, FIBP, HSPA8, KBTBD7, NCL, PARP1, RBBP6, and TCOF1 were confirmed to interact with NiV M protein.

Of these confirmed *Henipavirus* M-interacting host proteins, the binding regions within AP3B1, KBTBD7, and ARL6IP4 were mapped down to small polypeptides of about 100 amino acids in length. These peptides could be used to disrupt the virus-host protein-binding interface leading to inhibition of Nipah VLP production. The mapping of these binding regions also revealed a common characteristic between many of the host proteins identified; more than half of the NiV M-binding host proteins contained serine-rich acidic regions. The presence of the serine-rich acidic regions within so many of the host proteins identified suggests a potential importance for electrostatic interactions between *Henipavirus* M proteins and the host's cellular machinery. Together, this work has provided a solid platform to further our understanding of the host factors that are important for *Henipavirus* M protein function.

Our initial co-affinity purification screening of SH-HeV M resulted in the identification of numerous host proteins, of which a large fraction were known RNA-binding proteins (In preparation Sun, et al., unpublished). This was thought to have

occurred as a result of a non-specific linkage of HeV M protein to known RNA-binding host proteins via RNA interactions. To address this problem, cellular RNA was degraded prior to affinity purification of SH-HeV M protein, this resulted in the successful removal of the majority of the previously identified RNA-binding host proteins. Additionally, the non-RNA-binding host proteins that were confirmed to bind with HeV M protein were not eliminated during this procedure along with many new host proteins not previously identified. We believe this procedure has provided us with a more biologically relevant view of the host factors that are important for HeV M protein function. Thus, this technique has allowed for a more specific purification of a paramyxovirus matrix protein with a seemingly high propensity for cellular RNA-binding.

Although the direct binding of RNA with a *Henipavirus* M protein has not been demonstrated, RNA-binding is common among other matrix proteins including Ebola virus, HRSV, HIV-1 and influenza virus (48, 92, 129, 160). Although it is not clear what the exact purpose of all of these RNA-matrix protein interactions are, work with HIV-1 gag suggests that its RNA interaction acts as a chaperone allowing for proper targeting of HIV-1 gag to sites of assembly at the plasma membranes that contain the phospholipid phosphatidylinositol (4,5)-bisphosphate (PI(4,5)P₂) (1). Alternatively, the RNA-binding of influenza virus M1 protein is required for the formation and transport of ribonucleoprotein complex (RNP) (65, 90). Taken together with our evidence, it is likely that *Henipavirus* M proteins bind with cellular RNA, and possibly viral RNA, to facilitate virus particle assembly. However, further characterization of M-RNA interactions using gel shift assays will be required to fully understand the implications of this interaction.

Mapping the binding regions within host proteins required for virus assembly and budding has proven a useful tool for the identification of potential inhibitors of virus replication (30, 85, 94, 119). This has been best characterized in viruses that depend on interactions with ESCRT machinery for assembly and budding via specific late domain

binding motifs. However, *Henipaviruses* have not been conclusively shown to contain any previously-defined late domain sequences for recruitment of ESCRT and Nipah VLP production is also relatively unaffected by disruption of ESCRT machinery (118). Since *Henipavirus* particle production is not dependent on ESCRT, new peptide inhibitors of budding were developed here. Following mapping studies of the binding between NiV M and host proteins, three inhibitory peptides were derived from AP3B1, KBTBD7, and ARL6IP4 (In preparation Sun, et al., unpublished). These peptides were able to reduce Nipah VLP production when overexpressed in mammalian cells with NiV M protein. This reduction in Nipah VLP production suggests a role for these virus-host interactions in the assembly and budding of *Henipaviruses*. However, infection of stable cell lines expressing these inhibitory peptides with NiV will provide additional insight into the potential for these peptides to be used for the basis of future antiviral drug design.

Following a comprehensive sequence analysis of all the *Henipavirus* M-interacting proteins identified, more than 50 percent were found to encoded serine-rich acidic regions. Since the inhibitory peptides we identified here contain this type of negatively-charged region, we predict that it is likely that serine-rich acidic regions within these proteins have a propensity to serve as a binding surface for *Henipavirus* M proteins. These protein interactions are thought to occur through electrostatic interactions between basic regions of M protein with the negatively-charged phosphorylated serine residues in combination with the surrounding acidic residues. Furthermore, almost all of the *Henipavirus* M-interacting proteins containing serine-rich acidic regions contained a DSES amino acid sequence. A similar DSES sequence was found to be required for KBTBD7 binding to NiV M protein during mapping studies. Additionally, multiple amino acid substitutions in the region surrounding the DSES sequence in AP3B1 also disrupted binding to NiV M protein (In preparation Sun, et al., unpublished). Despite the high frequency of confirmed *Henipavirus* M proteins that

contain similar DSES sequences, there also seems to be alternative sequences that can facilitate electrostatic interactions with M protein independent of a DSES sequence.

Two proteins identified during sequence analysis, ARL6IP4 and NCL, did not contain a DSES sequence, but both proteins still contain potentially large negatively-charged binding surfaces. Instead, ARL6IP4 and NCL contain large stretches of likely phosphorylated serine residues or acidic residues, respectively. Although it is difficult to assign biological relevance to all of these virus-host protein electrostatic interactions, disruption of AP3 complex function through siRNA knockdown of AP3B1 or overexpression of AP3B1 inhibitory polypeptides can reduce *Henipavirus* particle production (In preparation Sun, et al., unpublished). Taken together, these results support a model where *Henipavirus* M proteins may use a common electrostatic interaction surface to bind with numerous host proteins for a variety of functions including: post-translational modification of M protein, trafficking of M protein, control of cellular transcription, and translation.

Of the many complexes identified during our screening of *Henipavirus* M proteins, several subunits of the translation initiation complex EIF3 were identified. The EIF3 complex normally functions to initiate cellular translation and as a result it provides an attractive target for viruses to manipulate. The rabies virus (RV) M protein has been implicated in regulating host protein translation through an interaction with the EIF3H subunit (79). Additionally, the EIF3 complex has also been observed to be important for both HCV and HIV-1 replication. The HCV internal ribosome entry site (IRES) is able to bind with the EIF3 complex for the purpose of its own translation initiation of viral mRNA, while HIV-1 requires interaction with the EIF3F subunit to aid in HIV-1 mRNA maturation (111, 140, 155). Although there has been no previously established links to the EIF3 complex for *Henipaviruses*, we can speculate that *Henipavirus* M proteins might regulate host protein translation by blocking the binding of interferon (IFN)-inducible proteins,

such as P56. P56 normally binds with the EIF3E subunit to down-regulate translation following IFN stimulation (66). Blocking an interaction between P56 and the EIF3 complex would allow for *Henipavirus* M proteins to potentially regulate cellular and/or viral translation by during the course of infection.

Two additional complexes related to post-translational modification were also identified during *Henipavirus* M protein screening: ubiquitin E3 ligase complex and casein kinase II complex. The ubiquitin E3 ligase complex is mainly associated with the polyubiquitination of substrate proteins for the purposes of protein degradation. In this work we have identified KBTBD7, which belongs to a group of proteins that contain both a substrate binding domain, kelch repeats, and an E3 ligase recruitment domain, BTB/POZ domain. Other members of this group are known for their linking of substrates with E3 ubiquitin ligases. We have demonstrated that KBTBD7 likely functions to link NiV M protein with the CUL3 E3 ubiquitin ligase (Appendix B). However, further investigation of the targeting of NiV M protein to this E3 ubiquitin ligase complex is required to identify how the polyubiquitination of NiV M protein might control protein levels during viral infection.

Interestingly, KBTBD6 does not bind with NiV M protein despite sharing a nearly 90 percent sequence identity with KBTBD7 (Appendix A). This finding suggests that a very specific sequence is required for binding with NiV M protein. One possible reason for KBTBD6's inability to bind with NiV M protein is that KBTBD6 contains multiple proline residues upstream from the serine-rich acidic sequence that are not present in KBTBD7 (Fig. A-2B). These proline residues in KBTBD6 potentially create a kink in the protein's structure, which may not be compatible with binding to NiV M protein. However, further work is required to better understand the requirements for binding of KBTBD6 and KBTBD7 with NiV M protein. With a better understanding of these differences, we may also gain additional insight into the requirements for interactions

between NiV M protein and a number of other host proteins that also encode similar serine-rich acidic sequences.

A second complex involved in post translation modification, casein kinase II complex, was also identified. Post-translational modification by casein kinase II typically results in the phosphorylation of the substrate protein. The importance of viral protein phosphorylation has recently been demonstrated for PIV5 M protein. PIV5 M protein was shown to require phosphorylation of a serine at amino acid position 369 for binding with the regulatory host protein 14-3-3 (120). Disruption of PIV5 M protein's interaction with 14-3-3 by mutating this serine amino acid residue resulted in deregulated particle production, which surprising manifested as an increase in virus particle budding. Alternatively, disruption of matrix protein phosphorylation can negatively impact virus assembly and budding; this is the case for the Ebola VP40. Preventing the phosphorylation of the tyrosine at amino acid position 13 resulted in reduced VLP production and a 4-log reduction in Ebola virus replication. These recent findings illustrate the potential functional importance of NiV M protein phosphorylation by casein kinase II complex.

There are numerous examples of other viral matrix proteins that require interaction with ESCRT for virus assembly and budding. However, *Henipavirus* particle production occurs independent of the ESCRT machinery (118), highlighting the need to identify novel host protein complexes required for the assembly and budding of *Henipavirus* M proteins. One promising complex that has been identified during screening is the adaptor protein complex 3 (AP3). We have observed that Nipah VLP production requires interaction with the AP3 complex where knockdown of endogenous levels of the AP3 complex subunit AP3B1 reduces VLP production (In preparation Sun, et al., unpublished). Additionally, the overexpression of the inhibitory peptides discussed earlier also reduces Nipah VLP production. Although our results suggest that the AP3

complex is important for Nipah VLP production it is unlikely that the AP3 complex is the only host factor required for NiV assembly and budding.

In addition to the AP3 complex, we have also identified several members of the profilin 1 complex. NiV M protein was confirmed to bind with HSPA8 also known as heat shock cognate protein 70 (HSC70). HSPA8 has been reported to be required for the transport of influenza virus genomes by M1 protein along with several non-assembly related functions in other virus systems (163). Based on the previously known functions of HSPA8 in other virus systems it is feasible that HSPA8 functions as a chaperone of NiV M protein that facilitates the trafficking of NiV M protein to sites of assembly and budding. Although many of the proteins identified in this work have been defined as members of large protein complexes, many are multifunctional and are also found in complex with other cellular machinery. Therefore, the distinct possibility exists that they may be functionally important for *Henipaviruses* in ways not discussed here. Only a directed investigation into each protein-protein interaction will be able to determine the biological relevance of these confirmed virus-host protein interactions.

Chapter 5

Summary and Future Directions

5.1 Summary

Cooperation between numerous viral and host factors are required for every step of the virus life cycle. During the final steps of virus particle production, it is common for viral protein to cooperate in order to form infectious virions. Previous studies with Sendai virus (SeV) protein have identified the viral C protein as a positive contributor to virus particle production. However, unlike SeV C protein, the Nipah virus (NiV) C protein was unable to enhance Nipah virus-like particle (VLP) production. Interestingly, vesicles containing NiV C protein were able to release into the media independently of other viral components. The release of NiV C protein containing vesicles was found to be dependent on a membrane-targeting sequence located in the N-terminus of the protein. The membrane-targeting sequence required a cluster of hydrophobic and basic residues to direct NiV C protein to membranes. This membrane-targeting sequence was also sufficient to redirect enhanced green fluorescent protein (EGFP) to intracellular membranes. The ability to bind with membranes seems to be a common characteristic of paramyxovirus C protein as both SeV C and human parainfluenza virus type 1 (hPIV1) C protein also bind with cellular membranes (96, 137).

It was also observed that NiV C protein was rapidly degraded due to a short half-life of 30 minutes. This protein degradation is dependent on the ubiquitin-proteasome pathway in that inhibiting the proteasome function prevents NiV C protein turnover. Analysis of NiV C protein's ubiquitination status discovered that NiV C protein was heavily ubiquitinated at several sites along the protein and that this ubiquitination was at least partially responsible for NiV C protein degradation. Interestingly, a mutant NiV C protein lacking basic N-terminal residues, NiV (+)8A.C protein, was stable despite still

being ubiquitinated. However, this observation is not fully understood and may indicate that the ubiquitination of NiV C protein might not only serve as a degradation signal. Taken together, we have defined several molecular characteristics of NiV C protein, which may play important roles in NiV replication and pathogenesis.

In addition to characterizing NiV C protein, yeast 2-hybrid and co-affinity purification screens were performed to identify *Henipavirus* M-interacting host proteins. As a result of these screens, twelve host proteins were confirmed to bind with NiV M protein in mammalian cells. For three of these NiV M-interacting proteins, AB3B1, KBTBD7, and ARL6IP4, we were able to create small NiV M-binding peptides. The overexpression of these binding peptides was able to inhibit Nipah VLP production by up to 3-fold. Furthermore, additional work has utilized siRNA knockdown of endogenous AP3B1 protein resulting in similar consequences for *Henipavirus* budding. Since all three of these proteins are expressed in a wide variety of cell types including the lungs (28, 64, 132), these binding peptides provide potential targets for novel antiviral drug design.

In addition to identifying numerous *Henipavirus* M-interacting proteins, a new method has also been developed to eliminate the non-specific effects of *Henipavirus* M-RNA interactions on affinity co-purification screening. Compared to a previous screen performed without RNase A treatment, the new method resulted in a 2.5-fold reduction in the number of co-purified RNA-binding proteins with *Henipavirus* M protein that strongly suggested that *Henipavirus* M protein binds to RNA. This could provide a useful tool for many other viral matrix proteins as many bind to RNA and would likely behave similarly (48, 92, 129, 160). The extensive screening performed here also provides a first step in the establishment of a *Henipavirus* interactome. To date there has not been any published work investigating this aspect of *Henipavirus* biology. To this end, we have worked to identify several potential host protein complexes that may play a critical role in

Henipavirus M protein function. These cover a wide variety of cellular processes including: transcriptional regulation, protein synthesis, post-translational modification and protein trafficking.

Following a comprehensive assessment of the amino acid sequences of M-interacting host proteins, we were able to identify that more than half contained similar serine-rich acidic regions. During mapping studies of KBTBD7 and AP3B1, we determined that clusters of negatively charged amino acids intermixed with potentially phosphorylated serine residues that served as likely regions of interaction with *Henipavirus* M proteins. Similar regions were identified in: AP3B1, EIF3C, KBTBD7, RBBP6 and TCOF1. The only two exceptions to this were ARL6IP4 and NCL. However, both ARL6IP4 and NCL contain striking stretches of more than twenty serine or acidic amino acids, respectively. These observations support a model where *Henipavirus* M proteins use electrostatic interactions with host proteins dependent on their temporal and spatial kinetics during assembly and budding.

5.2 Future Directions

In part, work presented here has identified several characteristics of NiV C protein, many of which are shared with SeV C protein. However unlike SeV C protein, no direct links have been made between the molecular and functional characteristics of NiV C protein. Arguably, one of the most important of these functional links may be between NiV C protein's molecular characteristics and its ability to disrupt the cellular interferon (IFN) signaling. The C proteins encoded by SeV and hPIV1 both bind directly only with STAT1 (43, 137). This leads to the degradation of STAT1 by inducing instability of the SeV C-STAT1 bound complex or by sequestering of STAT1 in perinuclear aggregates by hPIV1 C protein (137). We hypothesize that NiV C protein interacts with critical components of the IFN-signaling pathway to either induce

degradation of the protein complex or merely to prevent the proper activation of the signaling pathway. To this end, a yeast 2-hybrid screen was performed for NiV C protein similar to what has been described above for NiV M protein; however, none of the host proteins identified are known to be involved in the innate immune response (data not shown). It is known from previous reports that NiV C protein behaves as an IFN-antagonist (116, 174), although we were unable to detect any protein-protein interaction between NiV C protein and STAT1 (data not shown). Future studies should assess if expression of NiV C protein is able to prevent the translocation of activated STAT1 into the nucleus following IFN stimulation. Additionally, pulse-chase analysis should be conducted to determine if the protein stability of STAT1 is reduced in the presence of NiV C protein. These studies may provide insight into the mechanisms by which NiV C protein suppresses IFN-signaling and potentially provide a functional significance for some of the molecular characteristics of NiV C protein defined here.

Outside of a role for NiV C protein in inhibition of IFN-signaling, there is also evidence that NiV C protein can inhibit genome replication (141). This property is not unique to NiV C protein: the measles virus (MeV) C and V protein can also inhibit MeV genome replication (127). In addition, there is a functional overlap between these viral proteins as both NiV and MeV C proteins can inhibit each other's genome replication but not human parainfluenza virus type 3 (hPIV3) genome replication. It is postulated that the SeV C protein increases viral polymerase selectivity for replication of viral genomes at the cost of decreasing polymerase processivity (149). Future work should aim to characterize the defective forms of NiV C protein described here as they relate to NiV minigenome replication. Our NiV minigenome system can be used to determine if NiV C protein requires membrane binding or rapid protein turnover to regulate genome synthesis. This may provide some meaningful insight into the functional requirements

for the inhibition of *Henipavirus* genome replication and a broader understanding of the mechanisms of control that *Heinpariviruses* utilize during genome replication.

The membrane binding defective forms of NiV C protein were unable to release into the culture media. This release was also dependent on NiV C protein ubiquitination in that removing sites of ubiquitin attachment prevented the release of NiV C protein into the media. Similarly, cumulative removal of sites of ubiquitin attachment has been shown to cause budding defects in HIV-1 gag protein and PIV5 M protein (50, 55). One proposed hypothesis is that the monoubiquitination of viral proteins involved in budding can serve as a sorting signal for the cellular ESCRT machinery. This may also be the case for NiV C protein, as removal of sites of ubiquitin attachment from NiV C protein prevented its release into the media. To address this, we propose to directly fuse Ub to NiV C protein and derivatives that fail to release into the media to assess the relative importance of monoubiquitination as a sorting signal for NiV C protein. Further characterization of the role of monoubiquitination in NiV C protein release may provide insight into the interplay between ESCRT and NiV C protein release.

Alternatively, mutating a potential AIP1-binding sequence YPVL, encoded by NiV C protein, or overexpressing dominant-negative proteins that disrupt ESCRT function, can also be used to assess the ESCRT-dependence of NiV C protein's release. These strategies have employed identify specific links to ESCRT machinery in a wide variety of enveloped viruses. We hypothesize that an AIP1 binding motif, YP(X)_nL, encoded by NiV C protein, serve to bind with the ESCRT component AIP1. Preliminary evidence using pair-wise yeast two-hybrid binding assays suggest that there is a direct interaction between NiV C protein and AIP1 (data not shown). Since AIP1 is also commonly associated with the budding of intraluminal vesicles (ILVs) into multivesicular bodies (MVBs) and the budding of enveloped viruses from the plasma membrane, it is possible that an interaction between NiV C protein and AIP1 is required for the release of NiV C

protein containing vesicles. Further investigation into this interaction may provide a clear link to an ESCRT-dependent mechanism for NiV C protein release.

If in fact NiV C protein is released within exosomes, they may be biologically active and serve as a form of intercellular signaling by fusing with nearby cells following release. Given the known functions of NiV C protein in suppression of IFN-signaling and inhibition of inflammatory cytokine production (98, 174), transfer of NiV C protein to nearby cells may play a significant role in shaping the microenvironments where NiV replicates. This type of signaling has been observed before in the HIV-1 Nef protein. Nef was observed to release within cellular exosomes that could transport Nef into nearby uninfected cells in order to induce cell death (86). Similarly, the NiV C protein may also be incorporated into exosomes that fuse with distant or nearby cells. However, NiV C protein may not be released within exosomes via endosomal sorting pathways. Instead, NiV C protein may independently traffick to the plasma membrane and directly release into the media similar to the budding process of many enveloped viruses. Future studies should aim to identify the mechanism required for secretion of NiV C protein containing vesicles and to determine the potential for these vesicles to fuse with cell membranes to exert NiV C protein related effects.

Since we have observed that NiV C protein is degraded in a proteasome-dependent manner, and NiV C protein also requires ubiquitination for secretion into the culture media, it is reasonable to assume that there may be multiple types of ubiquitin attachment occurring on NiV C protein. We predict that monoubiquitination is likely required for the release of NiV C protein containing vesicles similar to the requirements of many enveloped virus gag and M proteins, while polyubiquitination of NiV C protein directs its proteasome-dependent degradation. However, the mechanisms that would direct these events are unknown. Since specific combination of E3 and E2 ubiquitin ligases are known to direct specific types of ubiquitin attachment, NiV C protein may be

able to recruit multiple combinations of E3-E2 ubiquitin ligases for either mono- or poly-ubiquitin attachment. Identification and characterization of the types of E3-E2 ubiquitin ligases required for ubiquitin attachment to NiV C protein will provide needed insight into how mono- or poly-ubiquitination dictates NiV C protein function.

During analysis of the membrane targeting sequence in NiV C protein we identified a derivative of NiV C protein, NiV (+)8A.C, that did not undergo proteasome-dependent degradation. As a result, NiV (+)8A.C protein accumulated to extremely high levels within transfected cells, although similar amounts of NiV (+)8A.C protein were secreted as compared with unmodified NiV C protein. Additionally, further investigation of NiV (+)8A.C protein found that despite being stable there were similar ubiquitinated species detected as compared with the unmodified NiV C protein (data not shown). These observations led to our hypothesis that there may be multiple factors that determine if polyubiquitinated NiV C protein is ultimately degraded by the proteasome.

To better understand why NiV (+)8A.C protein is not degraded despite being polyubiquitinated further investigation into the requirements for NiV C protein proteasome-dependent degradation is necessary. Some potential degradation requirements include: the unfolding of polyubiquitinated NiV C protein to allow for entry into the proteasome, de-ubiquitination of NiV C protein prior to proteasome entry, a failure of host proteins to recognize and traffick polyubiquitinated NiV C protein to the proteasome, and the recruitment of inappropriate E3 Ub ligases by NiV (+)8A.C protein resulting in alternate types of ubiquitin attachment. Any number of these mechanisms may contribute to the accumulation of polyubiquitinated NiV (+)8A.C protein that is unable to be degraded and incompatible with vesicle release. This line of research would not only shed light onto the requirements for NiV C protein degradation but also would make significant contribution to our collective understanding of the ubiquitin-proteasome system.

In addition to characterizing the viral protein requirements for VLP production we also identified numerous *Henipavirus* M-interacting host factors. Using binding information derived from the identified M-interacting host proteins, small peptide inhibitors were created which when overexpressed inhibited Nipah VLP production. We hypothesized that in addition to inhibiting Nipah VLP production, these small inhibitory peptides may also be potent inhibitors of replication during NiV infection. Previous work in our laboratory has demonstrated the potential of this approach using an inhibitory peptide derived from AMOTL1 to inhibit PIV5 replication from infected cells (119). To this end, stable cell lines that constitutively express peptides that inhibit NiV M protein budding are currently under development. Once created these stable cell lines will be sent to our collaborator Dr. Lin-Fa Wang at the Australian Animal Health Laboratory where they will infect these cell lines with NiV under BSL-4 containment. Alternatively, we could develop recombinant NiV harboring mutations in the M protein that do not allow for recruitment of the relevant host proteins. This approach may also provide valuable insight into the importance of these protein interactions. Taken together, these studies may not only provide insight into the relevance of these virus-host interactions during virus infection, but may also identify novel targets for antiviral drug design.

One significant challenge faced while developing small peptide inhibitors is their bioavailability and the method of delivery. One strategy to overcome the challenge of delivery of peptides is to fuse them to other cell-penetrating peptides or protein transduction domains (Reviewed in 59). We hypothesize that fusion of a cell-penetrating peptide to the inhibitory peptides identified here may allow for these peptides to enter cells and inhibit Nipah VLP production. One example of these cell-penetrating peptides is a minimal amino acids sequence derived from the HIV-1 Tat protein (158). This minimal HIV-1 Tat peptide has been commonly used to allow for delivery of peptides of interest into cells. However, other methods of peptide entry may also prove to be

effective methods of peptide delivery. Future investigation into the requirements for peptide delivery would provide a significant step forward in the development of novel therapeutics to treat NiV infections.

An alternative approach to yeast two-hybrid screening could also be used to identify cellular factors that are functionally important for NiV assembly and budding. One such approach could utilize a high-throughput luciferase based RNA interference (RNAi) screening assay. This system could take advantage of mapping studies of the Nipah M-N protein interaction interface where the region required for binding has been identified within NiV N protein (In preparation Ray, et al., unpublished). Fusion of this NiV N binding peptide to a luciferase reporter protein would allow for NiV M protein to specifically incorporate luciferase into Nipah VLPs. Nipah VLPs production efficiency could then be measured using highly sensitive high-throughput enzymatic assays following RNAi knockdowns. This alternative approach would provide direct functional evidence for the importance of specific host factors required for Nipah VLP production.

In an effort to improve a previous co-affinity purification screen of HeV M protein a new method was developed. During our initial co-affinity purification of HeV M protein, numerous RNA-binding proteins were identified, such as RNA helicases, splicing factors, and ribosomal proteins. The second round of screening was able to eliminate these co-purified RNA-binding proteins by degrading the RNA prior to purification, providing strong evidence that HeV M protein directly binds cellular RNA. It is not uncommon for viral matrix proteins to bind with viral and cellular RNA to aid in the assembly of infectious particles (1, 65). However, further investigation using gel shift assays would be able to provide confirmation of this interaction. Additional characterization of the contact points required for HeV M-RNA-binding, could be used to create RNA-binding defective HeV M protein mutants. Generating RNA-binding defective HeV M protein

mutants may reveal if RNA-binding is required for selection of viral genomes into virions or possibly the proper targeting of M protein to sites of assembly and budding.

A number of avenues exist for exploration of the interactions between *Henipavirus* M proteins and host protein complexes, many of which may modify M protein's structure and function. One such area requiring further study is how *Henipavirus* M proteins may regulate the EIF3 complex. The EIF3 complex is a large group of proteins that promote protein translation initiation. The measles virus (MeV) N protein and rabies virus (RV) M protein can both inhibit EIF3 activity through interactions with EIF3 complex subunits (79, 133). We propose that *Henipavirus* M proteins may also inhibit host protein translation in response to viral infections through direct interactions with EIF3 complex subunits. Two primary mechanisms have been proposed for this type of inhibition: 1) titration of EIF3 subunits away from the complex prevents assembly of the complex or 2) the direct binding of viral proteins to the EIF3 complex inhibits proper translation initiation. However, further investigation into the recruitment of EIF3 complex by *Henipavirus* M proteins is required in order to identify if this interaction potentially directs a previously undefined function for *Henipavirus* M proteins.

Proper post-translational modification of viral proteins can be critical for controlling a viral protein's fate. Two cellular complexes that potentially modify *Henipavirus* M proteins were identified during screening, an E3 ubiquitin ligase and casein kinase II (CKII) complex. Recently, another paramyxovirus, PIV5 M protein has been found to require phosphorylation on a C-terminal serine residue for proper regulation of virus assembly and budding (120). Another enveloped virus, Marburg virus (MARV), requires phosphorylation of its matrix protein VP40 for the recruitment of viral nucleocapsids to sites of assembly in order to produce infectious particles (78). Given the precedent set by other viral matrix proteins, we propose that the *Henipavirus* M

proteins likely require phosphorylation for particle assembly and budding. The targeting of potentially phosphorylated serine and threonine residues for mutation can initially be performed based on their predicted phosphorylation status using the bioinformatics tool at MIT scansite (scansite.mit.edu). The identification of potential *Henipavirus* M protein phosphorylation sites may provide new insight into the functional importance of M protein phosphorylation.

A second type of post-translational modification, ubiquitination, has also been identified and partially characterized following screening. There are several examples of viral M protein that require monoubiquitination for assembly and budding including: PIV5 M, NiV M, and HIV-1 gag proteins (50, 55, 162). However, here we have identified an E3 ubiquitin ligase, CUL3, that typically polyubiquitinates substrate proteins leading to protein degradation. The recruitment of this type of CUL3 E3 ubiquitin ligase poses the interesting possibility that it functions to control M protein levels using proteasome-dependent degradation. This is potentially beneficial to *Henipaviruses* since viral matrix proteins can be highly immunogenic and excessive levels of matrix protein may stimulate the host immune response. Alternatively when hijacked by M protein, the CUL3 E3 ubiquitin ligase may actually control the monoubiquitination status of M protein for assembly and budding. However, a further understanding of how this E3 ubiquitin ligase complex interacts with *Henipavirus* M proteins is required to provide any additional insight into this form of M protein regulation.

Many virus-host protein interactions have been identified in this work, however all of the secondary binding assays were performed in HEK293T cells. Although the use of HEK293T cells provided a convenient method for confirming these interactions, they may not have represented the most relevant cell type in regards to virus infections in humans. Further characterization of these virus-host protein interactions in cells types derived from primary sites of *Henipavirus* infection would yield additional information

about the relevance of these virus-host protein interactions during human infections. Additionally, these virus-host protein interactions could also be assessed in a cell lines derived from the natural host reservoir species of *Henipaviruses*, fruit bats. These lines of research would not only increase our understanding of some of the differences of how *Henipaviruses* interact with their hosts, but also how these interactions translate into differences in viral pathogenesis across multiple species and lead to novel strategies to disrupt *Henipavirus* infections when they do occur in humans.

Appendices

Appendix A: Interaction of KBTBD7 with paramyxovirus M proteins

Proteins containing a BTB domain specifically interact with the CUL3 E3 ubiquitin ligase to recruit substrate proteins for polyubiquitination and subsequent protein degradation (170). This recruitment of specific protein substrates is typically mediated by a diverse group of protein-interaction domains such as: kelch repeats, zinc fingers, and MATH domains (38). To further explore the scope of the KBTBD7-NiV M protein interaction, a co-immunoprecipitation assay was used to determine if KBTBD7 could interact with viral M proteins from other *Paramyxovirinae* genera. Interestingly, KBTBD7 was able to bind with the PIV5, MuV and SeV M proteins (Fig. A-1 and data not shown). This finding suggests a potential importance of this interaction across several genera of *Paramyxovirinae*. To determine the specificity of the KBTBD7-NiV M protein interaction, co-immunoprecipitation was used to test the binding of NiV M with another closely related BTB-domain containing protein, KBTBD6. Surprisingly, only KBTBD7 was able to bind with both NiV and HeV M proteins despite sharing greater than 90 percent amino acid sequence identity with KBTBD6 (Fig. A-2A). This result illustrates the high specificity of the kelch repeat substrate-binding domains for binding to *Henipavirus* M proteins.

Following identification of the region within KBTBD7 required for binding to NiV M protein, the C-termini of KBTBD7 and KBTBD6 were compared to identify any potential sources that may account for the difference in binding. Of the region required for binding with *Henipavirus* M protein, amino acids 653-665, there is only a single amino acid difference at position amino acid 662 where KBTBD6 contains a leucine instead of a phenylalanine in KBTBD7 (Fig. A-2B). Despite the presence of hydrophobic amino acids

at amino acid 662, the lack of the bulky aromatic ring KBTBD6 may not be compatible with binding to *Henipavirus* M proteins. Alternatively, the presence of two proline amino acids in KBTBD6 at positions 652 and 636 may prevent binding to NiV M protein. Often, proline residues can result in a kink in the protein structure due to the conformational rigidity of the residue. This may significantly alter the structure of the C-terminus of KBTBD6 to a conformation not compatible with binding to NiV M protein. However, further investigation is required to identify the critical differences between the substrate binding domains of KBTBD6 and KBTBD7.

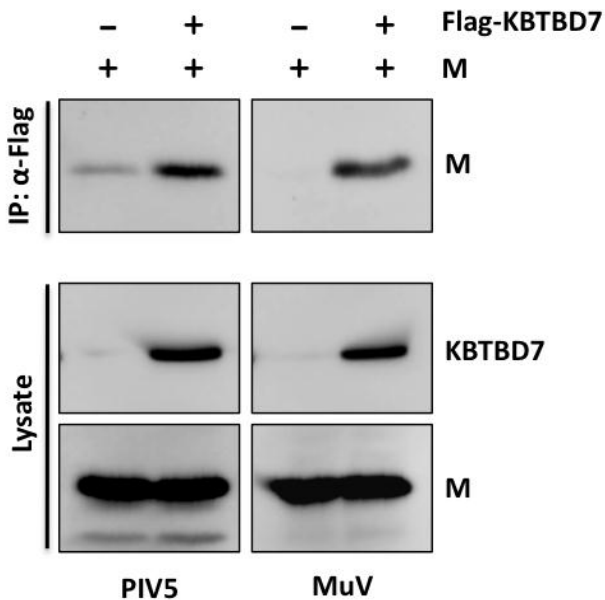
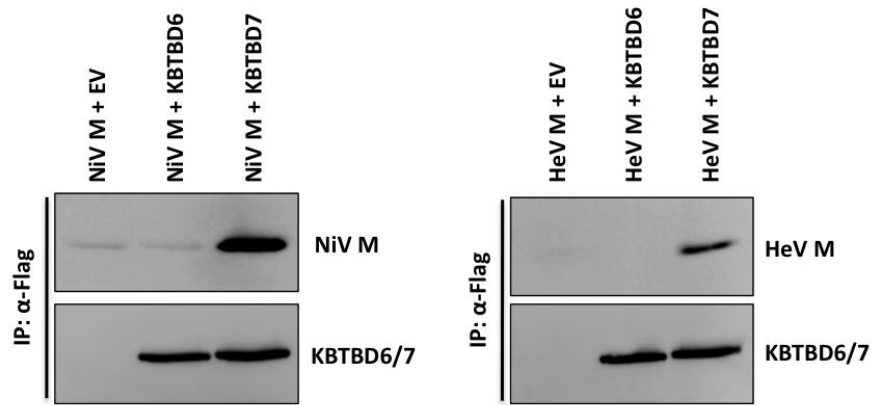


Fig. A-1 KBTBD7 binds with Rubulavirus M proteins.

HEK293T cells transfected to express PIV5 or MuV M proteins with Flag tagged KBTBD7 were lysed in co-immunoprecipitation lysis buffer containing 0.5% NP-40. Following lysis clarified cell lysates were immunoprecipitated with FLAG[®] magnetic affinity resin for 3h. Immunoprecipitated samples were resolved on 10% SDS-PAGE gels and subjected to immunoblotting with α-Flag, α-PIV5, or α-MuV M protein antibodies.

A



B

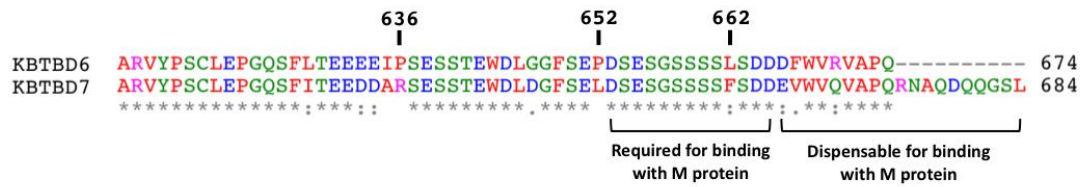


Fig. A-2 NiV M protein can only bind with KBTBD7 and not KBTBD6.

(A) HEK293T cells were transfected to express either NiV or HeV M proteins with either KBTBD6 or KBTBD7. Binding was measured as described previously in Fig. A-1. **(B)** A Clustal W sequence alignment of the C-termini of KBTBD6 and KBTBD7. The mapped region required for binding to NiV M protein is defined as being from amino acids 653-665. Three potential residues responsible for the lack of binding between KBTBD6 and NiV M protein are indicated: P636, P652 and L662.

Appendix B: KBTBD7 links NiV M protein to a E3 ubiquitin ligase CUL3

Recruitment of substrate proteins to E3 ubiquitin ligases such as CUL3 requires the interaction of a bridging factor containing a BTB domain and a substrate binding domain. One of the confirmed *Henipavirus* M-interacting host proteins identified here, KBTBD7, is predicted to contain both a BTB domain and a series of kelch repeats based on sequence homology to other known kelch and BTB domain containing proteins. During mapping studies, the kelch repeat containing C-terminus was identified as the region required for binding to NiV M protein. To further characterize these interactions, KBTBD7 and CUL3 were tested for pairwise binding with themselves and NiV M protein using co-immunoprecipitation assays. NiV M protein was confirmed to bind with KBTBD7, while only a weak co-immunoprecipitation of NiV M protein was detected with CUL3 (Fig. B-1A). As predicted, the pairwise testing of KBTBD7 and CUL3 identified a strong direct interaction between the two proteins (Fig. B-2B). Together these data establish the basis for interactions that may be occurring between these three factors during virus infections.

Additional binding experiments were performed to determine if KBTBD7, CUL3, and NiV M protein bind together as a complex that would be required for the ubiquitination of NiV M protein. The KBTBD7, CUL3, and NiV M proteins were expressed in HEK293T cells, by transient transfection. The pull-down of NiV M protein only led to a very weak co-precipitation of CUL3 when KBTBD7 was absent. However, when KBTBD7 was included it greatly enhanced the co-precipitation of CUL3 by NiV M protein (Fig. B-2A). Similarly, when CUL3 was immunoprecipitated in the presence of NiV M protein there was a weak detection of NiV M and it was only when KBTBD7 was included that NiV M protein was co-precipitated with CUL3 (Fig. B-2B). Based on these results, we propose a model where NiV M protein can recruit CUL3 E3 ubiquitin ligase

machinery via interaction with KBTBD7 for its ubiquitination. Future studies are required to determine if this complex leads to the ubiquitination of NiV M protein and how this modification might impact NiV M protein function during virus infection.

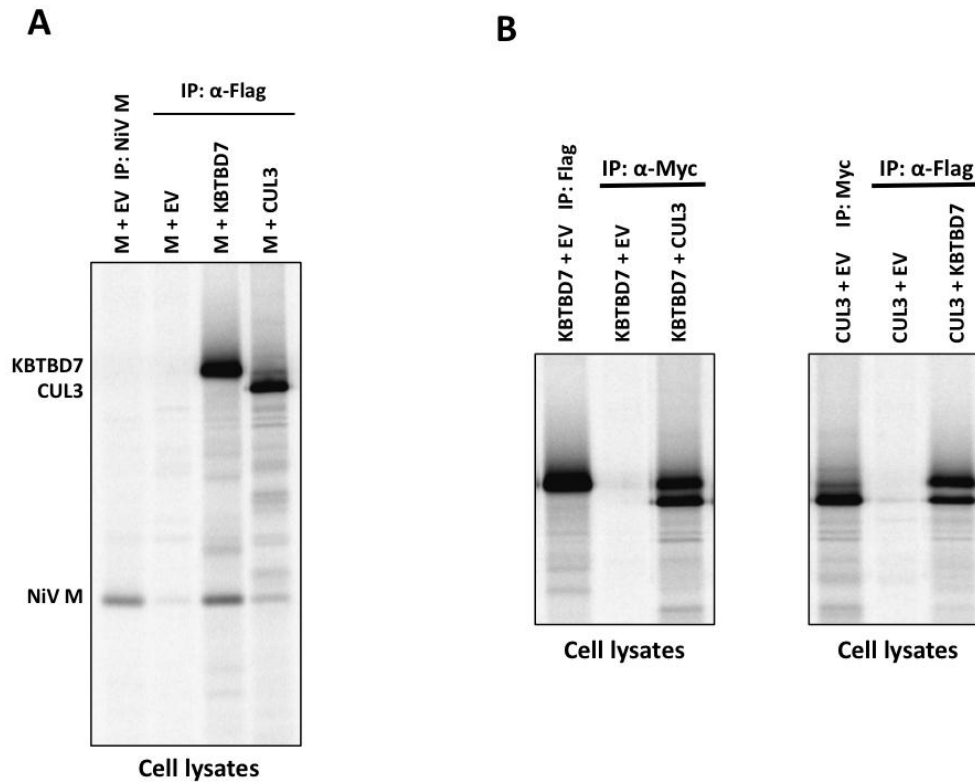


Fig. B-1 Pairwise interactions between NiV M, KBTBD7, and CUL3 proteins.

(A) HEK293T cells were transfected to express the indicated combinations of NiV M, KBTBD7 and CUL3 proteins. 24h p.t. cells were pulse labeled with ^{35}S for 3h before being harvest and lysed in co-immunoprecipitation lysis buffer containing 0.5% NP-40. The clarified lysates were immunoprecipitated with FLAG[®] magnetic affinity resin for 3h and loaded on 10% SDS-PAGE gels. Gels were exposed on a storage phosphor screen overnight and scanned on a Fuji FLA-7000 laser scanner. **(B)** The binding between KBTBD7 and CUL3 was measured as described above. Co-immunoprecipitations were performed using either α -Flag or α -Myc monoclonal antibodies as indicated.

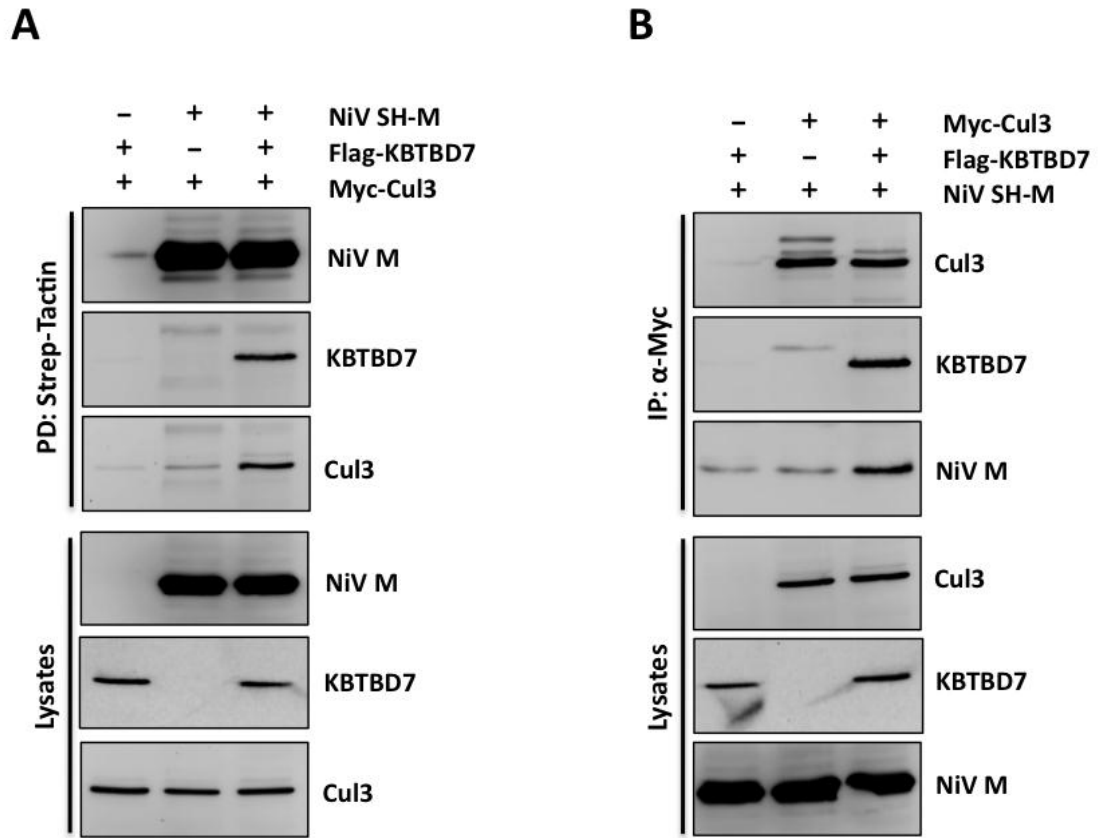


Fig. B-2 NiV M, KBTBD7, and CUL3 proteins interact as in a complex.

(A) HEK293T cells transfected to expressing SH-NiV M, KBTBD7 and CUL as indicated were lysed in co-immunoprecipitation lysis buffer containing 0.5% NP-40. Following lysis clarified cell lysates were pulled down with either Streptactin resin for 3h. Immunoprecipitated samples were resolved on 10% SDS-PAGE gels and immunoblotted with α -Flag, α -Myc or α -NiV M protein antibodies. **(B)** Binding experiment was performed as described above except co-immunoprecipitation was performed using the α -Myc monoclonal antibody.

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PUBLICATIONS AND PRESENTATIONS

Publications

- **McCrory, T.S.**, Schmitt, P.T., and Schmitt, A.P., 2012. Molecular Characterization of Nipah virus C Protein. (In preparation)
- Sun, W., **McCrory, T.S.**, Khaw, W.Y., Schmitt, P.T., and Schmitt, A.P., 2012. Viral and Host Protein Interactions During Henipavirus Assembly (In preparation)
- Li, M., Schmitt, P.T., Li, Z., **McCrory, T.S.**, He, B., and Schmitt, A.P., 2009. Mumps Virus Matrix, Fusion, and Nucleocapsid Proteins Cooperate for Efficient Production of Virus-Like Particles. *J. Virol.* **83**:7261-7272.

Posters and Presentations

- **McCrory, T.S.**, and Schmitt, A.P., "Molecular characterization of C protein encoded by a deadly paramyxovirus". Poster presentation. The Pennsylvania State University Graduate Exhibition, University Park, PA, March 2012.
- **McCrory, T.S.**, and Schmitt, A.P., "Role of Nipah virus C protein in virus budding". Poster presentation. 29th Annual Meeting of the American Society for Virology, Bozeman, MT, July 2010.
- **McCrory, T.S.**, and Schmitt, A.P., "Requirements for Nipah Virus-like Particle Production." Oral presentation. Pathobiology Summer Colloquium Series, University Park, PA, July. 2008;2009.