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ESCRT PATHWAY INVOLVEMENT IN THE RELEASE OF HUMAN PARAINFLUENZA VIRUS VIRIONS AND MATRIX PROTEIN

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ESCRT PATHWAY INVOLVEMENT IN THE RELEASE OF HUMAN PARAINFLUENZA VIRUS VIRIONS AND MATRIX PROTEIN

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ABSTRACT

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It has been shown that some enveloped viruses utilize cellular components to help facilitate viral budding and release. With these viruses, a viral protein interacts with the cellular ESCRT machinery, which is involved in the rearrangement of membranes and vesicle formation. Via this interaction, the virus directs the cellular machinery to form viral vesicles (particles) that bud from the infected cell. Little is known about the release of enveloped viruses belonging to the *Paramyxovirinae* subfamily and whether they utilize the ESCRT system. To better understand how human parainfluenza virus type 3 (HPIV3) mature virus particles are released from host cells an ESCRT inactivation system, developed with HIV-1, was used. Using siRNA targeting VPS4A/B, proteins essential for the recycling of ESCRT components, the levels of VPS4 protein in 293T and MK2-LLC cells were reduced by greater than 90%. These knocked down cells were then either transfected to express the HPIV3 Matrix protein or infected with HPIV3 whole virus. These results showed that even with the knockdown of VPS4, M-containing virus-like particles and virions were still able to be released from cells.

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TABLE OF CONTENTS

PAGE

LIST OF FIGURES……...vii

INTRODUCTION……...1

Taxonomy and Classification……..1

Clinical Features & Clinical Significance of HPIV3……...1

HPIV3 Structure……...3

HPIV3 Life Cycle……6

Attachment and Entry……..6

Transcription, Protein Synthesis, and Replication……...7

Assembly and Release…….7

Matrix Protein of *Mononegovirales*…….9

Late Domains…….10

ESCRT and MVB Pathways……..11

Late Domain Interactions with Cellular ESCRT Proteins…….13

ESCRT Pathway and Paramyxovirus Budding……..15

HPIV3 Matrix Protein……17

Late Domain of HPIV3 Matrix Protein…….18

HPIV3 Interactions with Alix……20

RESEARCH OBJECTIVES……..22

General Objectives…….22

Specific Objectives……22

MATERIALS AND METHODS……...23

RESULTS……..27

DISCUSSION……33

LITERATURE CITED……..37

LIST OF FIGURES

FIGURE PAGE

1. Phylogentic Tree of the *Mononegavirales*……...2
2. Schematic representation of HPIV3 virion structure and genome……...6
3. Life cycle of HPIV3…….8
4. Roles of ESCRT complexes in MVB biogenesis and virus budding…….13
5. Viral L domains found within viral proteins……..14
6. Trypsin digestion of M protein from media of transfected cells……...18
7. Budding of HPIV3 M protein with mutations in potential late domains……...19
8. Effects of Alix depletion on HPIV3 virion release……20
9. Knockdown of VPS4 in LLC and 293T cells……27
10. Effect of VPS4 knockdown in 293T cells on HPIV-3 Matrix protein release…...29
11. Effect of VPS4A/B knockdown in LLC cells on HPIV-3 viral release…….31
12. Average relative quantity of VPS4 and budding efficiency in LLC cells…….32

INTRODUCTION

Taxonomy and Classification

Human parainfluenza virus type 3 (HPIV3) belongs in the order *Mononegavirales*. HPIV3 along with the other viruses in this order are enveloped viruses that contain a negative sense, non-segmented RNA genome (1). This order is composed of five families, *Paramyxoviridae*, *Rhabdoviridae*, *Filoviridae*, *Nyamiviridae* and *Bornaviridae,* all of which contain important human and veterinary pathogens.

The *Rhabdoviridae* family contains viruses that can replicate in plants, invertebrates, or vertebrates (Fig. 1). Two common zoonotic pathogens in this family are rabies virus (RABV) and vesicular stomatitis viruses (VSV) (2, 3). The *Filoviridae* family only contains two genera *Marburgvirus* and *Ebolavirus,* whose viruses can cause severe viral hemorrhagic fevers. The third family is *Bornaviridae*, which contains Borna disease virus (BDV) (4, 5). BDV has a broad range of hosts but most commonly infects horses and sheep (6). The newest family added to the *Mononegavirales* order is *Nyamiviridae*, which contains tick-borne viruses that infect birds (7).

The final and largest family in the order *Mononegavirales* is *Paramyxoviridae*, which contains many clinically relevant viruses (Fig. 1). This family is split into two subfamilies *Paramyxovirinae* and *Pneumovirinae*, which are further spilt into seven and two genera, respectively. *Pnemovirinae* has two small genera the first being *Pneumovirus* which contains human respiratory syncytial virus (RSV), which is a common lower respiratory tract pathogen contracted during infancy, with most children

being affected by the age of two. The second genera is *Metapneumovirus* containing human metapneumovirus (HMPV), also associated with respiratory tract disease in humans (8).

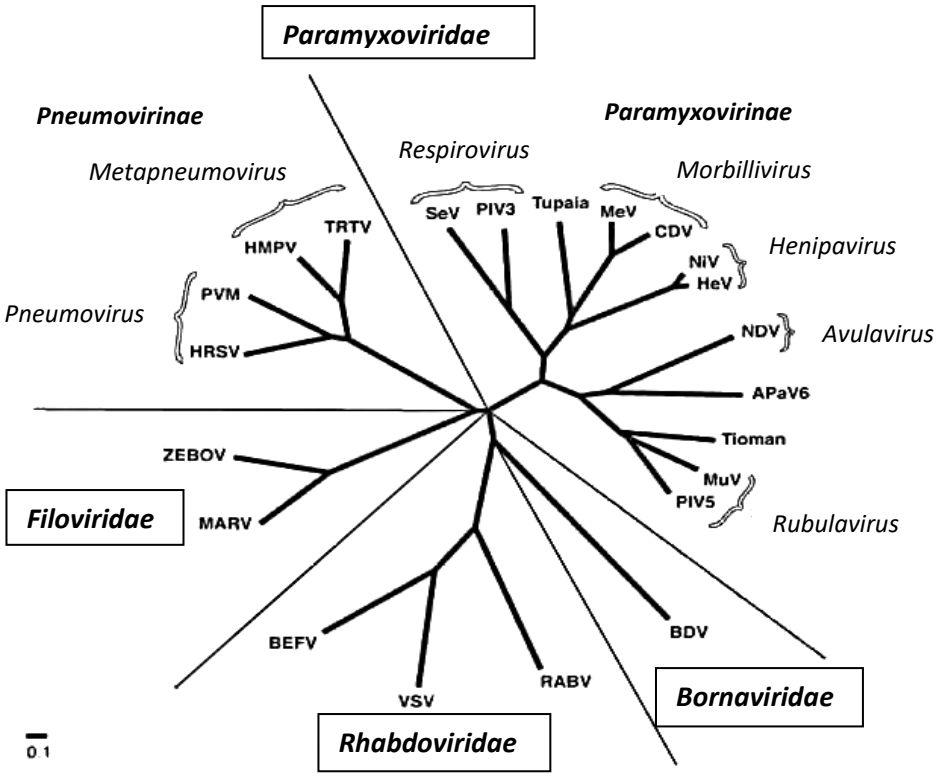

FIG. 1. Phylogentic tree of the *Mononegavirales* order showing important viruses from four families (boxed), sub-families (bolded, italics), and for the *Paramyxoviridae*, genera (italics). The tree was constructed using the CLUSTALX program with sequences of domain III of the polymerase proteins (1).

The second subfamily, the *Paramyxovirinae* contains seven genera with both human and animal disease-causing viruses. One of the most infectious viruses known is measles virus in the *Morbillivirus* genus. With an effective vaccine for measles viruses it is a target for eradication, but it is currently still prevalent in developing countries. The *Rubulavirus* genus contains viruses that cause generalized viral infections in children and adolescents, mumps virus (MuV), and two respiratory viruses, human parainfluenza virus

type 2 (HPIV2) and parainfluenza virus type 5 (PIV5). Two other parainfluenza viruses, human parainfluenza virus types 1 & 3 (HPIV1 & HPIV3) are within the *Respirovirus* genus. The prototypic virus of the family, Sendai virus (SeV), which infects rats and mice, is also in the *Respirovirus* genus. Two emerging viruses within the *Henipavirus* genus, Nipah virus (NiV) and Hendra virus (HeV), are zoonotic and have a broad tropism and have high virulence in humans compared to other paramyxoviruses (9).

Clinical Features and Significance of HPIV3

The parainfluenza viruses were so named because the disease characteristics and the shape and size of the virus particles were influenza-like. By 1960 there were four serotypes of human parainfluenza viruses recovered from pediatric patients suffering from respiratory tract disease (10, 11). Since then they have been recognized as important pathogens that cause acute respiratory illness worldwide. HPIV infections can cause a range of disease, from asymptomatic infections to lower respiratory diseases like croup, bronchiolitis and pneumonia (12). Most often, HPIV infections result in an upper respiratory tract infection, i.e. the common cold. However, HPIV infections are the second most likely cause of bronchiolitis and pneumonia in young children, with HPIV3 being the most common amongst the HPIVs (10, 13).

HPIV3, along with other parainfluenza viruses, are often acquired in community settings and are spread through large droplet aerosolization or contact with contaminated objects (10, 14). Outbreaks of HPIV3 infections occur seasonally, usually peaking during spring and early summer, but sporadic cases also occur. Young children, the elderly, and immunocompromised people are more prone to having more severe lower respiratory tract illnesses.

HPIV infections induce host systemic and local humoral responses along with cellular responses. Immunity to HPIV is mediated by serum antibodies, secretory antibodies, and major histocompatibility complex class I-restricted cytotoxic T lymphocytes (15–17). In infants, serum antibodies are attained through maternal antibodies, mostly during the lactation period. These antibodies remain present for approximately four months, after which the HPIV antibody levels start to decline (10). Although an immune response is elicited, reinfection can occur throughout life, usually presenting as an upper respiratory tract infection. Thus, the goal for several candidate vaccines for HPIV3 would be to lessen the severity of the first infection in infancy.

Many attempts have been made to create vaccines for HPIV3. Both inactivated and subunit vaccines have been tested with little to no protection created (11). The most promising candidates are live attenuated versions of the HPIV3 virus. One of these candidates is HPIV3 cp45 (cp = cold passaged) that was created through 45 passages in primary African green monkey kidney cells at low temperatures (18). The second candidate is bovine parainfluenza virus type 3 (BPIV3), which is naturally attenuated in humans (11). Both cp45 and BPIV3 have been tested in phase I and phase II trials in adults, HPIV3 seropositive children, HPIV3 seronegative children and infants as young as 1 month of age (11, 19). These candidates are still being tested and currently there are no FDA approved vaccines for HPIV3.

HPIV3 Structure

The virions of HPIV3 are medium-sized, pleomorphic particles having an average diameter ranging from 150-200 nm. Within the virion rests the single stranded, non-segmented, negative sense RNA genome of 15,462 nucleotides. The HPIV3 genome

encodes six structural proteins and one non-structural protein (Fig. 2). The genome is encapsidated by the viral nucleoprotein (N). This N-RNA complex then associates with the multifunctional large protein (L) and the phosphoprotein (P). The L and P proteins comprise the functional RNA-polymerase enzyme that binds to the negative sense RNA template. Together these three proteins and the genome make the P-L-N-RNA complex, which is termed the ribonucleoprotein (RNP) complex (10, 20, 21). The exterior of the virion is composed of a lipid bilayer envelope attained via the budding process from an infected host cell. The envelope is covered in surface projections that are made of fusion (F) protein homotrimers and hemagglutinin-neuraminidase (HN) protein tetramers (11). The glycoproteins F and HN are adhered within the envelope by a hydrophobic domain with the majority of the protein positioned exterior to the virion, and a small portion inside the viral particle (10, 12, 22, 23). The matrix protein (M) is located on the inner surface of the viral envelope (12, 23). The M protein is thought to have interactions with the carboxy terminus of the HN protein, the amnio terminus of the F protein, and the RNP complex (10). The matrix proteins of the paramyxoviruses have basic and hydrophobic properties and are the most abundant protein in virions (20, 24).

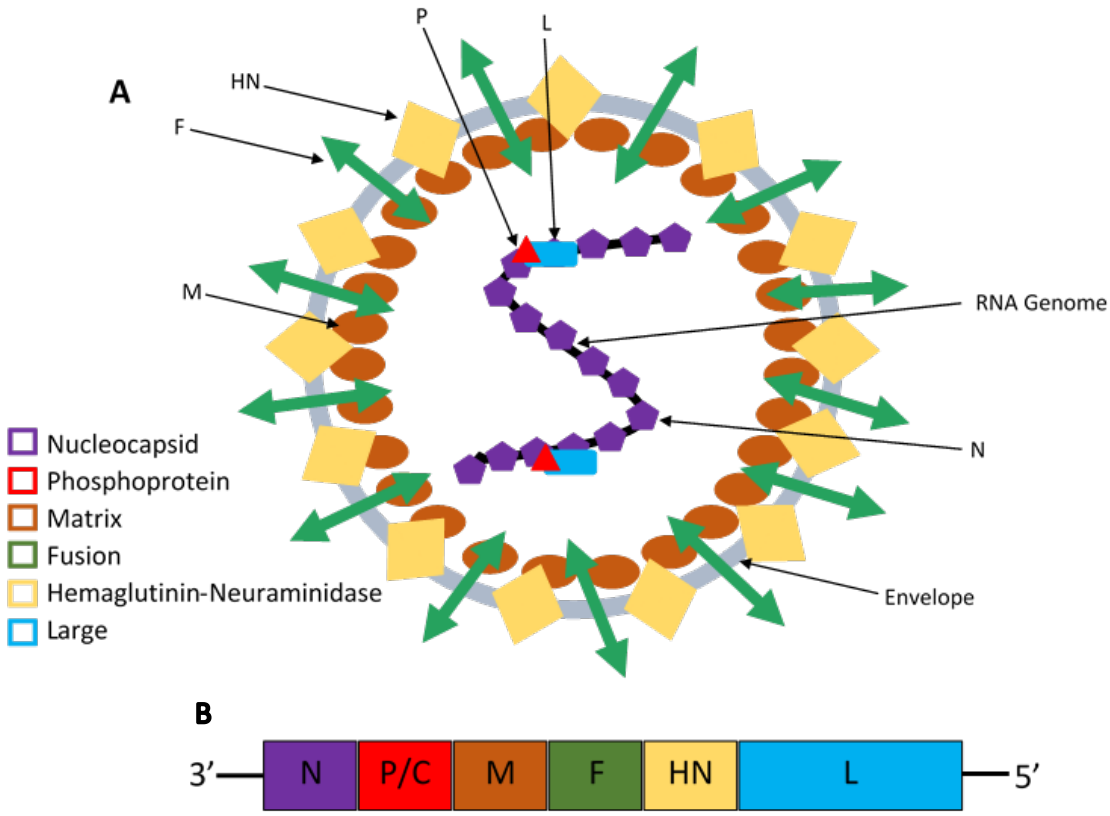

FIG. 2. A. Schematic representation of HPIV3 virion structure. **B**. Genome organization of HPIV3.

Life Cycle

Attachment and Entry

An HPIV3 infection begins by the attachment of virus particles to epithelial cells that line the respiratory tract (Fig. 3). Attachment is mediated by the binding of HN glycoproteins to sialic acid residues on the surface of host cells. Once the virions are bound to the sialic acid residues, fusion of the host cell plasma membrane and the viral envelope occurs via the F protein. Once the membranes are fused, the contents of the virion, including the RNP complex, is emptied into the host cytoplasm where the remainder of HPIV3's infection process occurs (10, 21, 24).

Transcription, Protein Synthesis and Replication

Upon entry of the RNP complex into the host cytoplasm, primary transcription begins. Transcription occurs through a sequential stop-start mechanism. The viral RNA-dependent RNA polymerase (vRDRP formed through the association of the P and L proteins) binds to the 3'end of the viral genome. Once bound to the genome it travels down the template, successively making six positive sense mRNA molecules that then serve as templates for the translation of the seven viral proteins using host ribosomes.

After primary transcription and translation have produced sufficient amounts of N protein, viral genome synthesis begins. Genome synthesis is coupled with the encapsidation of the positive-sense, "antigenomic" RNA. The antigenome is a replication intermediate and serves as the template for synthesis of genomic RNAs. The progeny genomic RNAs can be used as templates for additional transcription, further genome replication, or be packaged into virus particles.

Assembly and Release

After the syntheses of all viral components including the six structural proteins and the genomic RNA, particles start being assembled within the cell. One of the first steps in assembly is the formation of the N-RNA complex when the N protein binds the RNA genome during genome synthesis. Following this, the P and L proteins bind together and then to the N-RNA to form the RNP complex. The next step is for the assembly of viral components at the inner surface of the host cellular membrane. In this process, the M protein appears to bind and relocate the RNP complexes to locations on the plasma membrane that contain the viral F and HN proteins.

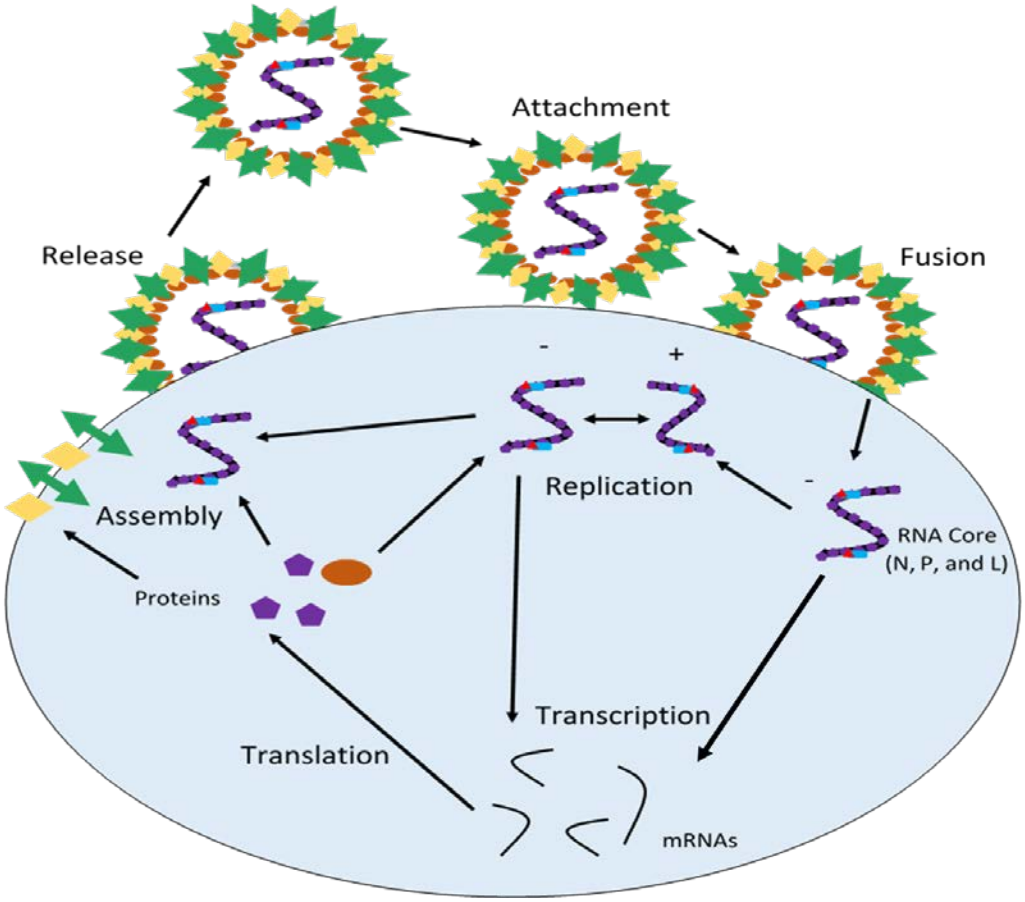

FIG. 3. Life cycle of HPIV3.

Once all of the contents for a virus particle are arranged at the cellular membrane, the release process begins. This process entails the lipid bilayer of the host membrane pushing outward until membrane scission occurs, allowing for the newly formed virus particle to separate from the membrane. Newly released virus particles disperse out until they interact with a new host cell to begin the infectious cycle again. The virus is able to spread through the help of neuraminidase activity of the HN proteins, which cleave sialic acid residues on the cell and virus surfaces, preventing attachment of new virus particles to already infected cells or other virus particles (17, 25).

Matrix Protein of *Mononegavirales*

The M protein of the *Mononegavirales* is known to have a leading role in mediating assembly and release of virus particles. The ability of the M protein to contribute to assembly of virus particles is fairly well understood, but less is known about the role of the M protein in the budding process. This is especially true for the paramyxoviruses.

The M protein is known to have multiple interactions during the assembly and budding process. There is evidence that shows that M proteins can self-associate, interact with nucleocapsid proteins, the cytoplasmic tails of the integral membrane proteins, and the lipid bilayer (10, 26–32). The assembly process is thought to involve the self-association of M in combination with its interactions with the N protein, and binding the cytoplasmic tails of either HN, F or both transmembrane proteins. The M-N, M-HN, and M-F protein interactions have been observed within multiple viruses, including members of the *Rhabdoviridae*, *Paramyxoviridae* and *Filoviridae* families (26, 27, 29, 31, 32). Multimerization of the M protein has also been observed within these families, in which different viruses form dimers, trimers, and/or larger multimers (33–37).

Interaction studies show good justification for M protein's role in assembly, but the evidence for M involvement in the budding process of the *Mononegavirales* is best understood through M protein expression studies. When the M protein is expressed alone, in the absence of other viral proteins, it is able to produce virus-like particles (VLP), which are composed of host cell lipid membrane surrounding viral M protein (16, 17, 20, 38–41). This budding process has been observed for HPIV-1, MeV, VSV, NDV, Nipah virus (NiV), HPIV-3 and EbV (17, 20, 37, 38, 42–46). This evidence strongly

suggests that the M proteins of paramyxoviruses are the primary facilitators of virus particle release.

Late Domains

The M proteins of viruses of the *Mononegavirales* order are able to release from host cells in the form of enveloped VLPs, but the mechanisms for which it does so are not clearly understood (47). For retroviral Gag proteins and some *Mononegavirales* M proteins, highly conserved short amino acid sequences play a critical role in the budding and release of virus particles from host cell plasma membranes. These sequences are termed late domains to indicate a function (budding) that is late in the viral life cycle. These highly conserved motifs are known to mediate protein-protein interactions between viral and cellular proteins which redirect the cellular proteins to the host plasma membrane where viral assembly and release occurs. At this site the cellular proteins assist in the budding and release process of the virus.

The initial evidence for this late domain function arose from studies on the HIV-1 Gag protein, which has a role similar to the M protein of the *Mononegavirales*. When viruses containing a deletion of a 6 kDa region of the HIV-1 Gag protein were tested, the virus particles failed to detach from infected host cell plasma membranes, stopping the viral life cycle (48, 49). It was found that a short peptide sequence (PTAP) was responsible for the release of HIV-1 particles from the host cell plasma membranes (50). Further investigation revealed that retroviruses use three distinct classes of late domains to successfully release virus particles, which were: Pro-Thr/Ser-Ala-Pro (PT/SAP), Tyr-Pro-Xn-Leu (YPx,nL), or Pro-Pro-X-Tyr (PPxY) (where X represents any amino acid

and n is either 1 or 3) (28). These findings were groundbreaking as prior hypotheses proposed that release of enveloped virus particles was solely driven by viral factors.

After the identification of the late domains in retroviruses, three viral late domain classes (P(T/S)AP, PPXY, and YPXL) were found within the *Mononegavirales* order, specifically in the *Filoviridae* and *Rhabdoviridae* families (48). Within the *Paramyxoviridae,* late domains have been suggested, but none of the potential late domains exactly match the three already established domain sequences. Proposed late domains in NiV(62-YMYL- 65) and SeV (49-YLDV-52) may be variants of the YPxL late domain (43, 51), and a proposed late domain in PIV5 (20-FPIV-23) appears to be novel and not similar to any of the previously described domains (52).

ESCRT and MVB Pathways

It has been shown that late domains function by binding cellular proteins normally involved in cytokinesis, the formation of vesicles and membrane rearrangements (30, 32, 53). The cellular proteins involved in these events are termed endosomal sorting complex required for transport (ESCRT) proteins. In cells, the ESCRT machinery is involved in the manipulation and reorganization of lipid bilayers during many cellular processes. One example of the function of the ESCRT machinery involves degrading and/or repurposing integral membrane proteins to help maintain cell homeostasis. Cell surface proteins that are intended for degradation are tagged to be recognized by the ESCRT machinery through mono-ubiquitination. These ubiquitylated membrane proteins are internalized into an endosome. Then, on the surface of endosomes, the tagged proteins are internalized when vesicles push into the lumen of the endosome, creating intraluminal vesicles (ILVs). The modified endosome, called a multivesicular body (MVB), can fuse

with lysosomes so that the cargo proteins within the ILVs are degraded by lysosomal proteases (16, 47, 54–60). The ESCRT pathway includes 5 distinct protein complexes (ESCRTs-0, -I, -II, -III, -IV) and an assortment of other factors that each serve a different function. Early acting ESCRT complexes (ESCRT-0, -I, and –II) assemble mainly within the cytoplasm, and the late acting modules (ESCRT-III and IV), function specifically on the membranes. The ESCRT complexes in eukaryotic cells have additional functions including fusion of MVBs to lysosomes and separation of cells during cytokinesis (54–56, 61).

The ESCRT complexes are sequentially recruited to endosomal membranes in order to mediate membrane fission away from the cytoplasm. The assembly of the ESCRT complex begins with the recruitment of the ESCRT-0 to cellular membranes through binding ubiquitinated transmembrane proteins (59). ESCRT-0 then sequentially recruits the ESCRT-I complex via protein-protein interactions. Once ESCRT-I is recruited it engages ESCRT-II, which then nucleates ESCRT-III. The activated ESCRT-III complex is able to polymerize on lipid bilayers forming helical structures allowing for membrane invagination (59, 62, 63). The VPS4 ATPase subunit of ESCRT-IV is thought to disassemble the ESCRT-III complex in order to recycle its subunits. VPS4 uses energy from ATP to remodel the ESCRT-III filaments and allow them to be released from the endosome membrane so that ESCRT-III can then be used for further rounds of vesicle formation (Fig. 4) (59, 64–66). The release of the ESCRT-III filaments occurs simultaneously as the scission step where the donor membrane is completely separated from the vesicle, the mechanism for which this occurs is not fully understood (59).

Thus, in relation to virus particle budding, it appears that some enveloped virus bind cellular ESCRT proteins through their late domains. These interactions allow the viral proteins to redirect the ESCRT machinery to the plasma membrane to initiate virus budding (away from the cytoplasm) and assist in virus release.

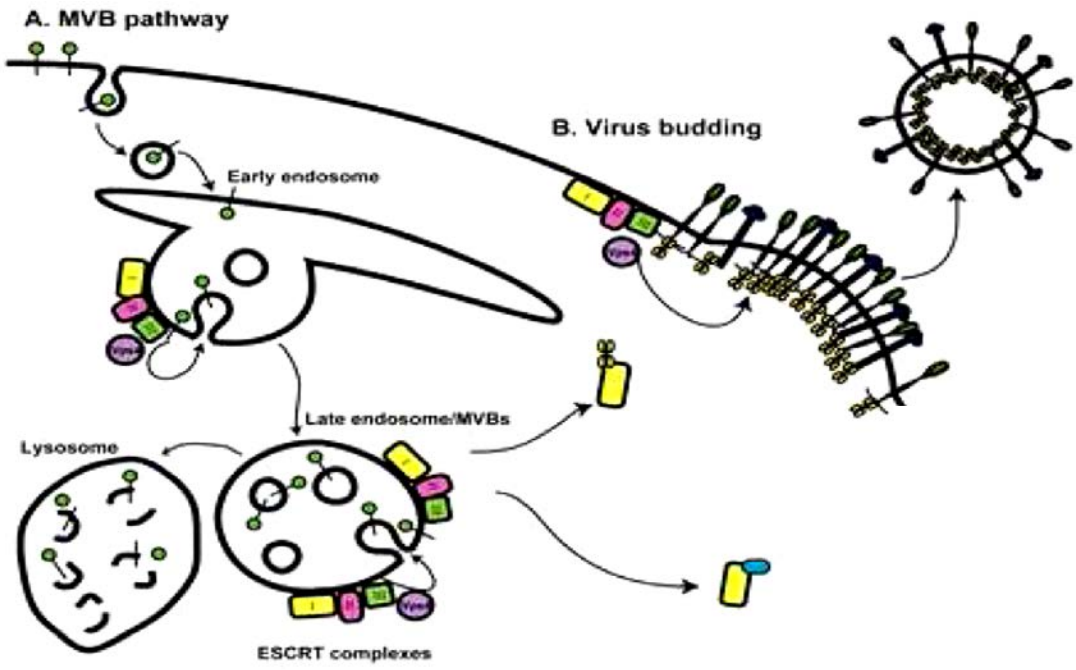

FIG. 4. A. Roles of ESCRT complexes in MVB biogenesis and virus budding. (=ESCRT I, =ESCRT II, = ESCRT III, = VPS4). (56)

Late Domain Interactions with Cellular ESCRT Proteins

Budding of enveloped viruses is most often coupled with viral assembly, and many such viruses use their structural proteins to recruit the ESCRT complexes to the site of viral budding to facilitate vesicle release (virus particle). It is now known that different enveloped viruses use at least four different classes of late assembly domains, whose functions are sometimes interchangeable. (Fig. 5).

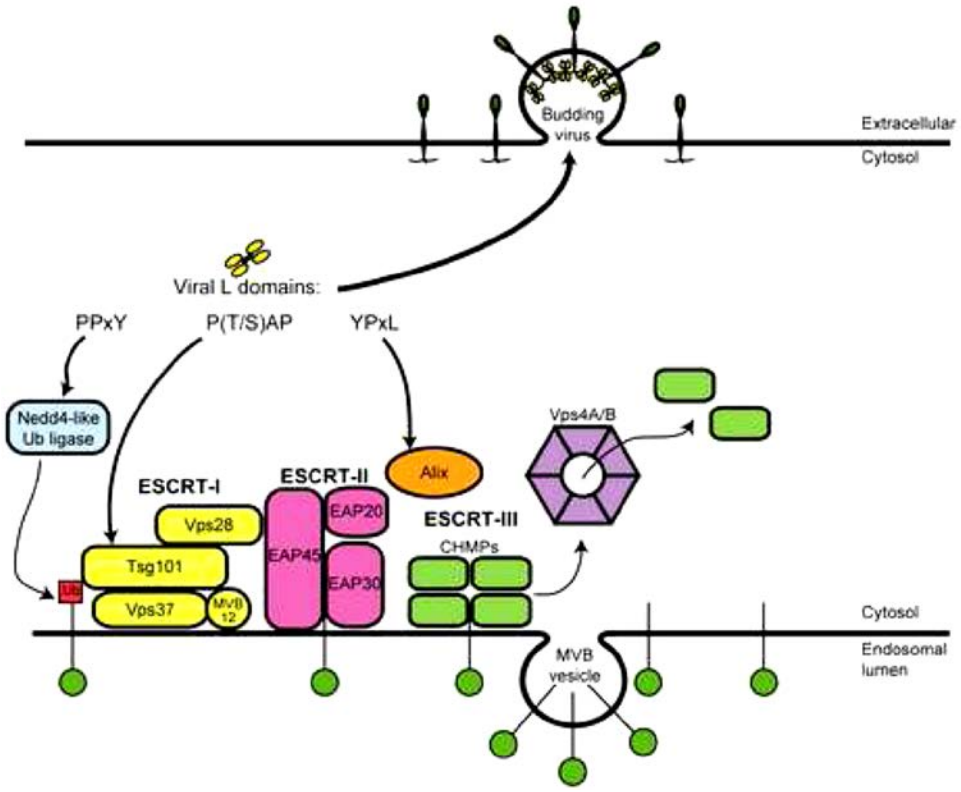

FIG. 5. Viral L domains found within viral proteins interact with components of the ESCRT complex redirecting them to the site of viral budding on plasma membrane (56).

The P(T/S)AP late domain functions by directly binding TSG101(Tumor susceptibility gene 101), which is a subunit of ESCRT-I, and recruiting it to the viral assembly sites (67–70). The YPxL motif binds ALIX (apoptosis-linked-gene2 product interacting protein X), which is not a component of an ESCRT complex (71–76), but is an adaptor protein that has direct interactions with the TSG101 subunit of ESCRT-I and the CHMP4 (charged multivesicular body protein 4) subunit of ESCRT-III, thus forming a bridge between the ESCRT complexes (73, 74, 77).

The PPxY motifs bind to the NEDD4 family of HECT E3 ubiquitin ligases (78–80). It is not well understood how the NEDD4 family proteins incorporate ESCRT machinery, but it is thought that ubiquitylation of NEDD4 substrates as well as ubiquitin

transfer is important for NEDD4 family dependent viral budding. As previously mentioned, unbiqutinated proteins are targeted for degradation via the MVB pathways, therefore ubiquitination of viral matrix proteins containing the PPxY motif could be a possible means through which a virus recruits the ESCRT machinery (28, 55, 56, 77, 81).

Lastly there are some additional "assembly" domains that do not strictly follow any of the above mentioned processes. In some paramyxoviruses potential late domains have been identified, showing a potential need for the ESCRT pathway. Although there have been no established ESCRT protein binding partners to these potential late domains; thus it is still unclear if these are true ESCRT recruiting late domains.

Late domain-mediated interactions with cellular ESCRT proteins have been described for some members in the *Filovirus* and *Rhabdovirus* families of the *Mononegavirales*. However the involvement of cellular proteins in the budding of members of the *Paramyxoviridae* is not well defined. The next section delves into what is known about the interactions with paramyxoviruses and the ESCRT pathway and its complexes.

ESCRT Pathway & Paramyxovirus Budding

Paramyxovirus M proteins lack any of the previously established late domain classes, but a few alternative sequences have been identified. These sequences have been shown to be important for viral budding activity, but the host factors with which they interact have been difficult to find. One particular domain has been found in PIV5 and mumps (52, 82). This putative late assembly domain has a consensus sequence of ØPxV (where Ø is an aromatic residue) that stimulate ESCRT-dependent virus budding (52, 82). However, this domain (FPIV in PIV5) is not technically classified as a

late domain due to the fact that no ESCRT binding partner has been identified. This leaves questions unanswered as to whether and how the ESCRT pathway is utilized in the viral budding of PIV5 and mumps.

Other sequences found to be important for paramyxovirus budding include YMYL in NiV(38) and YPLGVG in both NiV and HeV M (83). Although these sequences have been found to be important for viral release, the ESCRT components that would be associated with these sequences have yet to be identified. SeV, via a YLDL late domain, is the only paramyxovirus virus that has been shown to have binding interactions between an M protein and an ESCRT protein. The YLDL sequence is believed to be a variant of the YPXnL late domain because it appears to mediate binding of the SeV matrix protein to the ALIX protein(51)

In mammalian cells the cytosolic protein ALIX acts as an adaptor in the ESCRT pathway, binding proteins in ESCRT-I and III. During virus budding, YPXnL late domains on viral proteins bind to ALIX, thus recruiting the ESCRT machinery. The interactions between ALIX and its viral late domain motifs have been well characterized for retroviral Gag proteins (75) and proven to be essential for some viruses to effectively exit the host cell (71, 73, 75). SeV is the only member of the paramyxoviuses for which an interaction between the M (or any viral protein) and ESCRT complexes has been demonstrated. The interaction of SeV M and ALIX has been shown to correlate with virus budding (51, 53). However, contradicting evidence has also been published with SeV, showing that SeV does not need ALIX for viral budding (84). Since SeV is the best characterized paramyxovirus and it is closely related to HPIV3 in the *Respirovirus* genus,

understanding what is known about SeV release is valuable for our investigation of HPIV3.

It has been shown that expression of the SeV matrix protein in the absence of other viral proteins is effective in producing SeV-VLPs (17, 41). An analysis of the SeV M protein revealed the sequence 49-YLDL-52 to be a critical motif for SeV-VLP budding. Thinking that the YLDL sequence could possibly function as a YPXnL late domain, investigators looked at the idea of possible links between SeV budding and the YPXnL interaction partner in the ESCRT pathway, ALIX.

The first group to look at this interaction was the Sakaguchi group which found ALIX to be involved in the VLP budding process since a 90% decrease in SeV-VLP release was observed after depletion of ALIX with siRNA. They also observed binding of the ALIX protein to the SeV M protein to be dependent on the presence of YLDV domain. Soon after these findings, another paper was published by the Roux group, which also looked at the role of ALIX in SeV budding (84). Roux found that depletion of ALIX by siRNA did not decrease the release of SeV particles from infected cells. Both groups approached the question of the need for ALIX in slightly different manners, but neither have been able to provide a clear reason for the discrepancy in their results. However, one difference was that Roux looked at actual virus release while Sakaguchi looked at VLP release. The discrepant results could be explained if VLP release was more sensitive to ALIX depletion than virus particle release.

Human Parainfluenza Virus Type 3 (HPIV3) Matrix Protein

To comprehend the means by which HPIV3 undergoes its assembly and release process our lab began by characterizing the HPIV3 proteins involved in these actions.

The ability of HPIV3 M protein to trigger VLP formation was first determined by developing a functional budding assay. Expression of the HPIV3 M wt protein in transiently transfected 293T cells resulted in release of M-containing VLPs from the cells, indicating that HPIV3 M protein can be released from cells in the absence of any other viral proteins. To confirm that released M protein is in the form of a VLP, the media from cells expressing M protein was treated with Triton X-100, a membrane solubilizing detergent, and/or trypsin protease. M protein was only digested by trypsin in the presence of Triton X-100, demonstrating that the released M was most likely protected by an envelope membrane, therefore confirming that HPIV3 M protein was released from transfected cells as a VLP (Fig. 6).

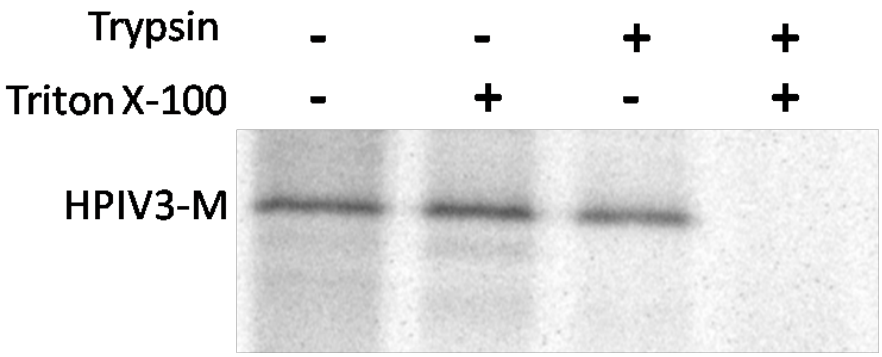

FIG. 6. Trypsin digestion of M protein from media of transfected cells with and without Triton X 100. (85).

Late Domain of HPIV3 Matrix Protein.

Since late domains in matrix proteins of the *Mononegavirales* were identified and functional, the lab analyzed HPIV3's M protein sequence for possible late domains. Four candidate sequences were identified: 47-PPKH-50, 54-YLDV-57, 300-YPLMDL-305, and 338-YPNI-341, and were selected for further testing. Of the selected sequences only one, YPLMDL, matched one of the established late domains. Additionally, the YLDV

sequence of HPIV3 aligns with the 49-YLDL-52 late domain of the close relative SeV. The other two potential motifs only differed from established late domains by conserved amino acid substitutions.

To test the functionality of these motifs as possible late domains, alanine substitution mutations were created to disrupt their possible function. The budding activities of these mutants were then tested through a budding assay. The most drastic change on M VLP release was seen via alteration of the YLDV and YPLMDL motifs. The APLMDL and ALDV mutants showed a significant decrease in efficiency when compared to the wt (Fig. 7).

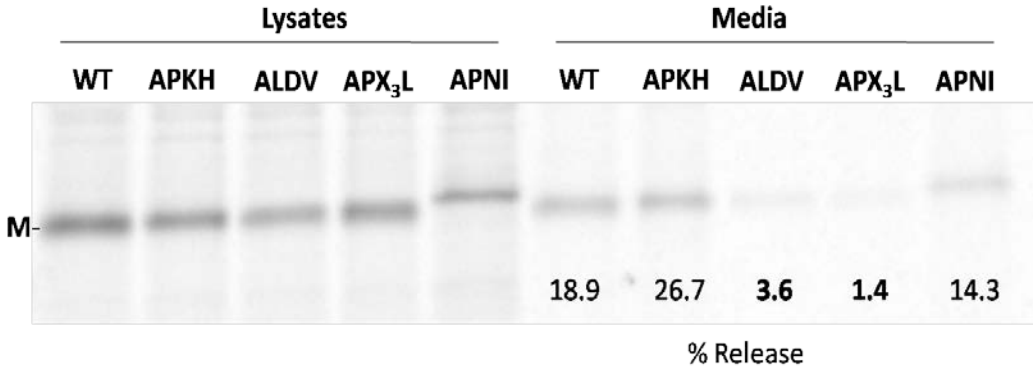

FIG. 7. Budding of HPIV3 M proteins with alanine substitution mutations in candidate late domains compared to M wt (Somboun Xiong unpublished data).

Thus, two sequences, 54-YLDV-57 and 320-YPLMDL-325, appear to be important for release of HPIV3 M protein VLPs and therefore could be late domains. Both of these possible late domains fall under the YPXnL motif type, which has been shown to bind the cytosolic protein ALIX (63, 72, 75, 76). However, we do not believe that both potential late domains actually function as late domains because they do not appear to be functionally redundant. If ALIX is recruited via either of the two potential late domains, then alteration of one should not significantly reduce budding efficiency

since the second late domain should remain functional. It is possible that one of these domains function as a late domain while the other serves another function important in VLP formation. Still, based on this observation, and by comparison to SeV which has been shown to utilize ALIX/ESCRT, we hypothesized that HPIV3 M protein uses the ESCRT complexes to facilitate budding from host cells.

HPIV3 Interactions with ALIX

With this knowledge our lab decided to pursue the question of whether HPIV3 needs ALIX for the budding process to be completed. The role for ALIX was looked at by creating cell lines with siRNA-mediated knockdown of ALIX which were then infected with HPIV3 to later observe virus particle release levels. It was shown that when ALIX was depleted from cells it had no apparent effect on the ability for HPIV3 to bud out of cells (Fig. 8). (56)

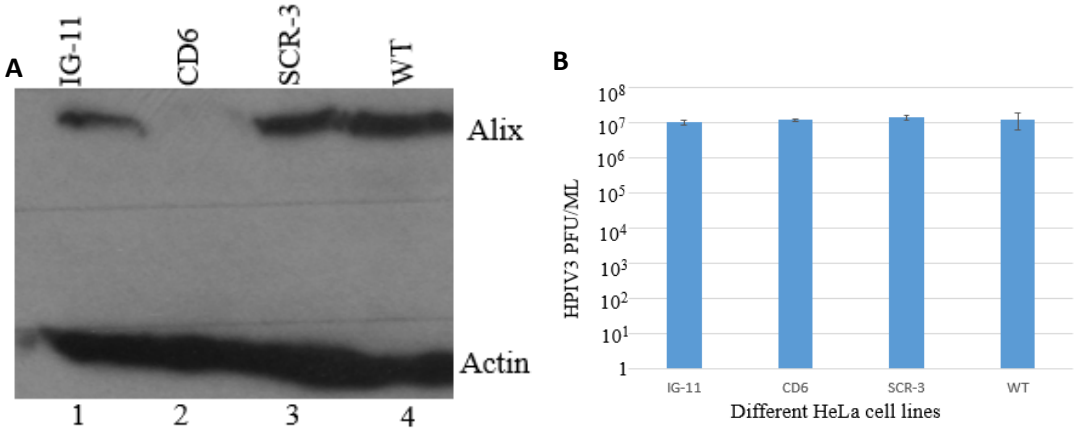

FIG. 8. A. Western blot displaying cell lines depleted of ALIX using siRNA. B. Effect of ALIX-knockdown in HeLa cells on HPIV3 particle release. At 24 hr post-infection, sample media was taken and titered. The bar graphs represent the titers of HPIV3 on different HeLa (IG-11 and CD6 Alix depleted; WT and SCR3 control) cell lines. Standard errors were calculated from two independent experiments. (Suresh Kandel unpublished data)

Although the YPXnL consensus sequence is a well characterized late domain that mediates binding to ALIX, it does not represent the only viral mechanism to recruit the ESCRT machinery. As discussed earlier, other components of the ESCRT pathway can be recruited in order for viruses to utilize the pathway's membrane altering capabilities, which could help account for the viruses' ability to bud without the presence of ALIX. Another thing to note is that siRNA can only knockdown a protein, synthesis is not completely stopped within a cell. Therefore, if only a small amount of ALIX is needed for viral budding, it is possible there was enough present for the ESCRT pathway to be properly recruited. To fully determine if the ESCRT pathway is used in the process of HPIV3 budding, other key proteins within the pathway can be examined to determine if the ESCRT pathway is needed for virus release.

RESEARCH OBJECTIVES

General Objectives

The ability of matrix proteins to direct the formation of VLP's is already clearly established. Though several motifs have been identified as being critical for HPIV3 VLP formation, how these motifs function in VLP formation is not understood. With recent findings from our lab showing that HPIV3 can bud efficiently with low levels of the ALIX protein, it raises the question of whether the ESCRT pathway is used at all. To clearly understand whether HPIV3 uses the cellular ESCRT pathway during particle assembly and release, other proteins within the ESCRT pathway must be viewed for importance. The final step of releasing the ESCRT III complexes from the lipid membrane and the newly formed virus particle from the cell is completed through the use of the ATPase VPS4. When VPS4 is not present the ESCRT III complexes are not released from the membrane preventing their further use by the ESCRT pathway and thus shutting down ESCRT-dependent processes. By focusing broadly on the role of the ESCRT pathway, we should be able to determine the significance of these proteins in paramyxovirus release.

Specific Objectives

1. Determine if the ESCRT pathway is utilized in HPIV3 budding
    A. Knockdown VPS4 in tissue culture cells
    B. Determine if the knockdown of VPS4 has an effect on HPIV3 budding or VLPs and release of virus particles

MATERIALS AND METHODS

Cells

293T (human embryonic epithelial kidney) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), and grown at 37ºC with 5% CO_2. LLC MK2 cells were cultured the same as described for 293T cells, except the DMEM was only supplemented with 5% FBS.

Plasmids

The pCAGGS M plasmids were previously constructed by inserting the HPIV-3 M protein DNA sequence into pCAGGS eukaryotic expression vectors by Michael Hoffman (85). Previously described siRNA duplexes targeting VPS4A (CCGAGAAGCUGAAGGAUUAdTdT) and VPS4B (CCAAAGAAGCACUGAAAGAdTdT) were purchased from Dharmacon (86, 87).

Virus-Like Particle Budding Assays

Transfections were performed with 25-30% confluent 293T cells in 12-well plates. The siRNA duplexes were added to 100 µl Opti-MEM™ (Gibco™ by Thermo Fisher Scientific) and 3 µl Lipofectamine RNAiMAX and incubated for 15 min at room temperature prior to being added to the cell culture media. At 24 hr post initial transfection, M plasmids were added to 120 µl Opti-MEM™ (Gibco™ by Thermo Fisher Scientific) and 3 µl X-tremeGENE™ 9 DNA transfection reagent (Roche) at room temperature and incubated for 15 min, prior to being added to the cell culture media. An additional transfection of the siRNA duplexes was also done under the conditions stated

above at 24 hr post initial transfection. The amounts used for each transfection were as follows: 50 pmol of VPS4A, 50 pmol of VPS4B, 25 pmol of Non-Targeting siRNA Control, 0.8 µg of M plasmid.

Samples were collected at 72 hr post initial transfection. To check for M proteins released from cells media was collected and centrifuged at 2,567 RCF for 10 min. The supernatant was then subjected to pelleting through a sucrose cushion by layering it on top of 3 mL of 20% sucrose in phosphate-buffered saline (PBS) (8 mM Na_2HPO_4, 137 mM NaCl 2mM Kh_2PO_4, 2.7 mM KCl) and centrifuging at 121, 570 RCF for 2 hr to pellet VLP's. Pellets were resuspended in 20 µl of 3X SDS running buffer (250 mM Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, 8% β-mercaptoethanol, 0.02% bromophenol, Boston BioProducts). To examine M protein inside cells, cells were resuspended in 400 µl of 1X PBS, centrifuged at 2,567 RCF, and lysed in 25 µl of 1X cell lysis buffer (25 mM Tris-phosphate, 2mM dithiothreitol (DTT), 2 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 1% Trition-X100, pH 7.6), and centrifuged for 5 min at 9,495 RFC; after 7 µl of supernatant was added to 5 µl of 3X SDS running buffer. For detection of proteins by western blot, the media and lysates were heated at 95ºC for 5 min. Proteins were separated by SDS-PAGE in 0.75 mm, 10% acrylamide gels. Proteins were transferred from the gel to Immobilon®-P polyvinylidene difluoride (PVDF) (Milipore®) membranes with a semi-wet transfer system at 0.25 volts and 0.25 amps for 28 min. After the transfer, membranes were blocked overnight at 4ºC with 5% milk in tris-buffered saline with Tween® 20 (TBST) (150 mM NaCl, 50 mM Tris-Cl, 0.05% Tween® 20, pH 7.5). After blocking, to detect HPIV-3 M protein, membranes were incubated with a polyclonal rabbit anti-M antibody in TBST with 5% milk (1:1000

dilution, Miceala Hass, unpublished data) for 1 hr. Membranes were then washed 3 times with TBST with 5% milk for 5 min, followed by three more 5 min washes with TBST. After washing, the membranes were incubated with donkey anti-rabbit HRP antibody (1:1000, Pierce) in TBST with 5% milk for 1 hr. The membranes were then washed 3 times with TBST with 5% milk for 5 min and then 3 more times with TBST for 5 min each.

To detect VPS4, membranes were incubated with a monoclonal anti-VPS4 antibody in TBST with 5% milk (1:250, Santa Cruz Biotechnology) for 1 hr. Membranes were then washed 3 times with TBST with 5% milk for 5 min, followed by 3 more 5 min washes with TBST. After washing the membranes were incubated with goat anti-mouse HRP antibody in TBST with 5% milk for 1 hr. The membranes were then washed 3 times with TBST with 5% milk for 5 min and then 3 more times with TBST for 5 min each.

To detect actin, membranes were incubated with a monoclonal anti-actin antibody in TBST with 5% milk (1:250, pan Ab-5, NeoMarker/Thermo ACTN05) for 1 hr. Membranes were then washed 3 times with TBST with 5% milk for 5 min, followed by 3 more 5 min washes with TBST. After washing the membranes were incubated with goat anti-mouse HRP antibody in TBST with 5% milk (1:500, Southern Biotech) for 1 hr. The membranes were then washed 3 times with TBST with 5% milk for 5 min and then 3 more times with TBST for 5 min each.

Following the secondary antibodies, all membranes were exposed to SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific), and visualized with a ChemiDoc™ Touch (Bio-Rad®) imaging system. All images were

analyzed with Image Lab™ (Bio-Rad®). The budding efficiency was found by comparing the amount of Matrix protein released into the media from the virus like particles to the total amount of Matrix protein found in the lysate and in the media.

Analysis of HPIV3 Release

To determine the effect of the VPS4 depletion on viral protein release, 40-50% confluent LLC MK2 cells were transfected with siRNA as stated above. At 24 hr post initial transfection, the cells were infected with HPIV-3 at multiplicity of infection (MOI) of 5 virus particles per cell. After the media was added a second transfection of siRNA was added as stated above.

Samples were collected 72 hr post transfection. Both media and lysates were processed as stated above for the VLP assay. They were then heated at 95ºC for 5 min, then subjected to SDS-PAGE on a 0.75 mm 10% acrylamide gel. The gel was then transferred to a membrane and immunoblotted according to the protocol for detecting VLPs, except instead of using polyclonal rabbit anti-matrix antibody, a polyclonal rabbit anti-RNP antibody (Courtesy of the Amiya Banerjee lab at Cleveland Clinic) was used for detecting M and HN+N proteins. The budding efficiency was found by comparing the amount of M and HN + N proteins released into the media from the virus particles to the total amount of M and HN + N proteins found in the lysate and in the media.

RESULTS

A. Objective 1A: Knockdown VPS4 in tissue culture cells

To determine if VPS4 protein is important for HPIV-3 budding, the VPS4 protein was knocked down in both 293Ts and LLC-MK2 cells through the use of siRNA duplexes targeting VPS4A and VPS4B. Cells were transfected twice with a combination of VPS4A and VPS4B siRNAs, or a non-specific scrambled control (SCR) siRNA at times 0 and 24 hr. Lysates were collected 72 hr post initial transfection and immunoblotted with an anti-VPS4 antibody. Both cell lines showed a decrease in the presence of VPS4 protein after exposure to VPS4 siRNA duplexes (Fig. 9).

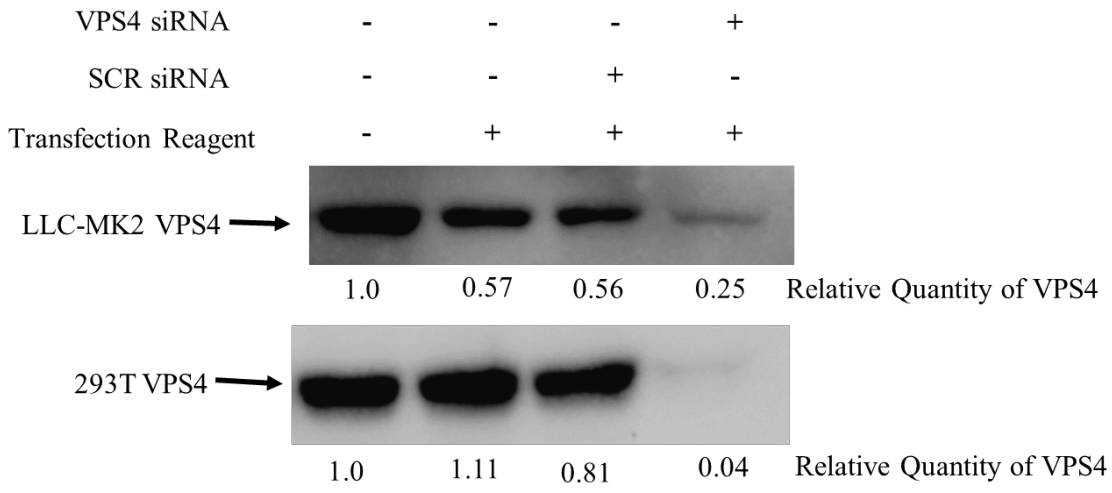

FIG. 9. Knockdown of VPS4 in LLC MK2 and 293T cells. LLC MK2 and 293T cells were transfected with siRNAs at 0 and 24 hours. Lysates were collected 72 hr post initial transfection and VPS4 was visualized by western blotting with an anti-VPS4 antibody.

After developing the procedure to have a consistent knockdown of at least 80% of VPS4 in both LLC MK2 and 293T cell lines, the effects of VPS4 knockdown on M VLP release and HPIV3 protein release in 293T and LLC MK2 cell lines, respectively.

Objective 1B: Determining if the depletion of VPS4 has an effect on HPIV3 budding & release.

To determine the effect of depletion of VPS4 in 293T cells on HPIV3 matrix protein-directed VLP formation, VPS4 was knocked down using siRNA duplexes as done above. In addition, the cells were transfected with an HPIV3 matrix-encoding plasmid 24 hr after the initial siRNA transfection. At 72 hr post the initial transfection, lysates and media samples were processed for viral protein analysis. The knockdown of VPS4 was substantial in 293T cells. Compared to mock transfected cells, expression was reduced by 99%, and compared to cells transfected with scrambled siRNA, expression was reduced 98% (Fig. 10). When VPS4 is knocked down, a 44% decrease in the budding efficiency of HPIV3 Matrix protein was observed, when compared to cells transfected with scrambled siRNA. Of note, there also appears to be an increase in M protein synthesis when VPS4 is knocked down (Fig.10). The budding efficiency normalized by the amount of actin, was also calculated. This gave budding efficiencies of 24.4% for Lane 2, 45.1% Lane 3, 46.5% Lane 4, and 17.5 % Lane 5. When the budding efficiencies are normalized it shows that the effect of knocking down VPS4 on VLP release is even less with only about a 30% decrease in VLP release when comparing Lane 2 to Lane 5.

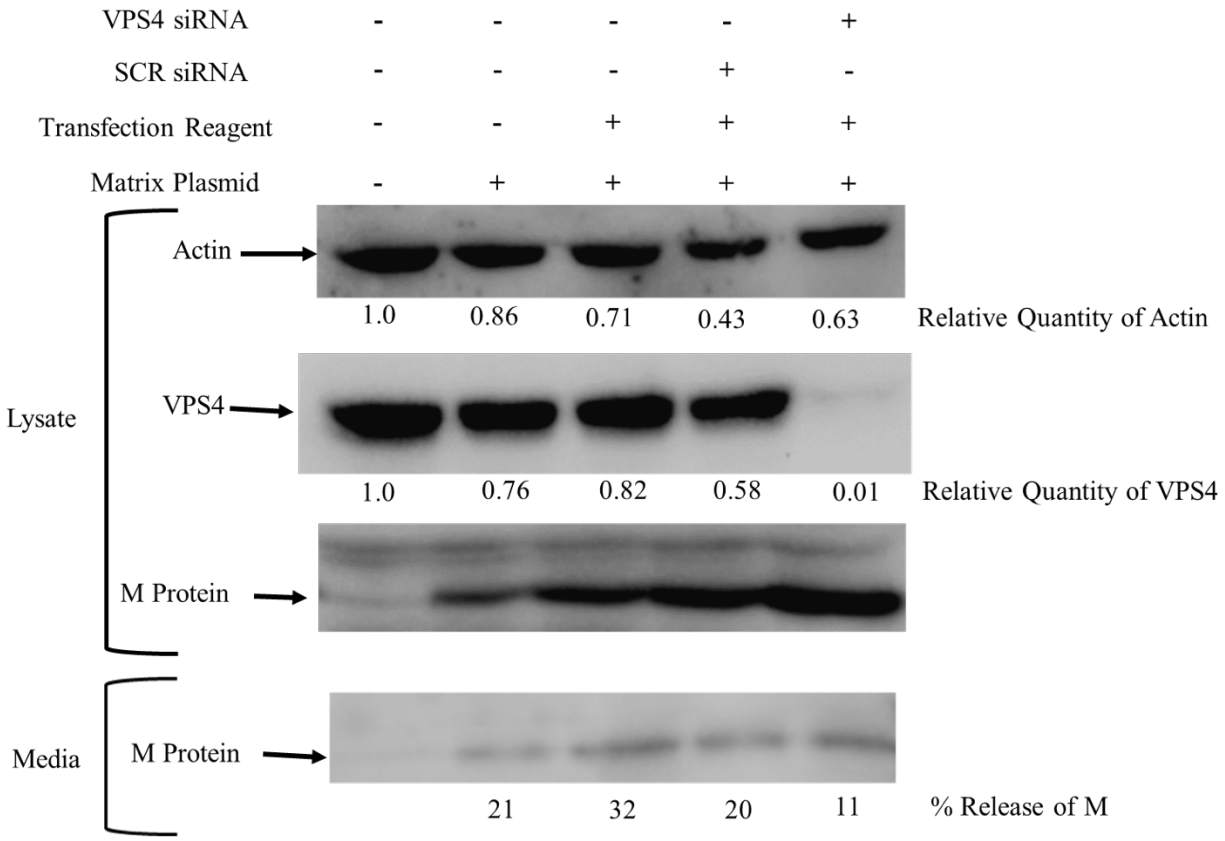

FIG. 10. Effect of VPS4 knockdown in 293T cells on HPIV-3 Matrix protein-directed VLP release. 293T cells were initially transfected with siRNA, and then at 24 hours post initial transfection, siRNA and 0.8 µg of M plasmid were transfected. At 72 hours post initial transfection media and cell lysates were harvested, and the media was subjected to ultracentrifugation through 20% sucrose. Proteins from both the media and lysates were separated via SDS-PAGE, and western blotted.

To further investigate the involvement of VPS4 in the HPIV3 lifecycle, studies with infectious virus were done. VLP assays work well in 293T cells, but HPIV3 does not grow well in this cell line. To look at the effects of VPS4 knockdown on HPIV3 virus particle production, LLC MK2 cells were used because HPIV3 replicates well in these cells.

To determine the effect of depletion of VPS4 on HPIV3 release from LLC-MK2 infected cells, VPS4 was knocked down using siRNA duplexes as previously described.

Specifically, siRNAs were transfected at 0 hr, then at 24 hr the cells were infected with HPIV3 at a MOI of 5. Also at 24 hr a second transfection of siRNAs was done. Samples were collected 72 hours post the initial transfection (48 hr post-infection), lysates and media were processed for viral protein analysis. This experiment was repeated numerous times because viral budding was not consistently detected. Furthermore, considerable cytopathic effects and fewer cells were observed in the transfected and infected cells. The displayed results were the best representation of viral budding while conducting this experiment (Fig.11). When examining actin levels, a decrease in actin was observed when transfection reagent was added, which was consistent with direct visual observation of increased cytopathic effects in these cells and decreased number of cells.

Additionally, in the particular experiment shown in Fig. 11, the actin protein levels were unusually low in the cells receiving the scrambled siRNA. The unusually low actin levels in the scrambled siRNA lysate lanes were not seen in other repetitions of this experiment. Thus, the best comparison of the effect of VPS4 depletion on virus protein release is made by comparing cells receiving transfection reagent and HPIV3 (column 3) to cells receiving transfection reagent, VPS4 siRNA, and HPIV3 (column 5). In comparing these lanes, there was a 67% decrease in VPS4 levels, while HN+N release decreased from 46 to 33%, and M release decreased from 49% to 39%. In this case the budding efficiencies were not normalized to actin due to the fact that the original numbers were inconsistent leading to the normalized numbers to be unreliable.

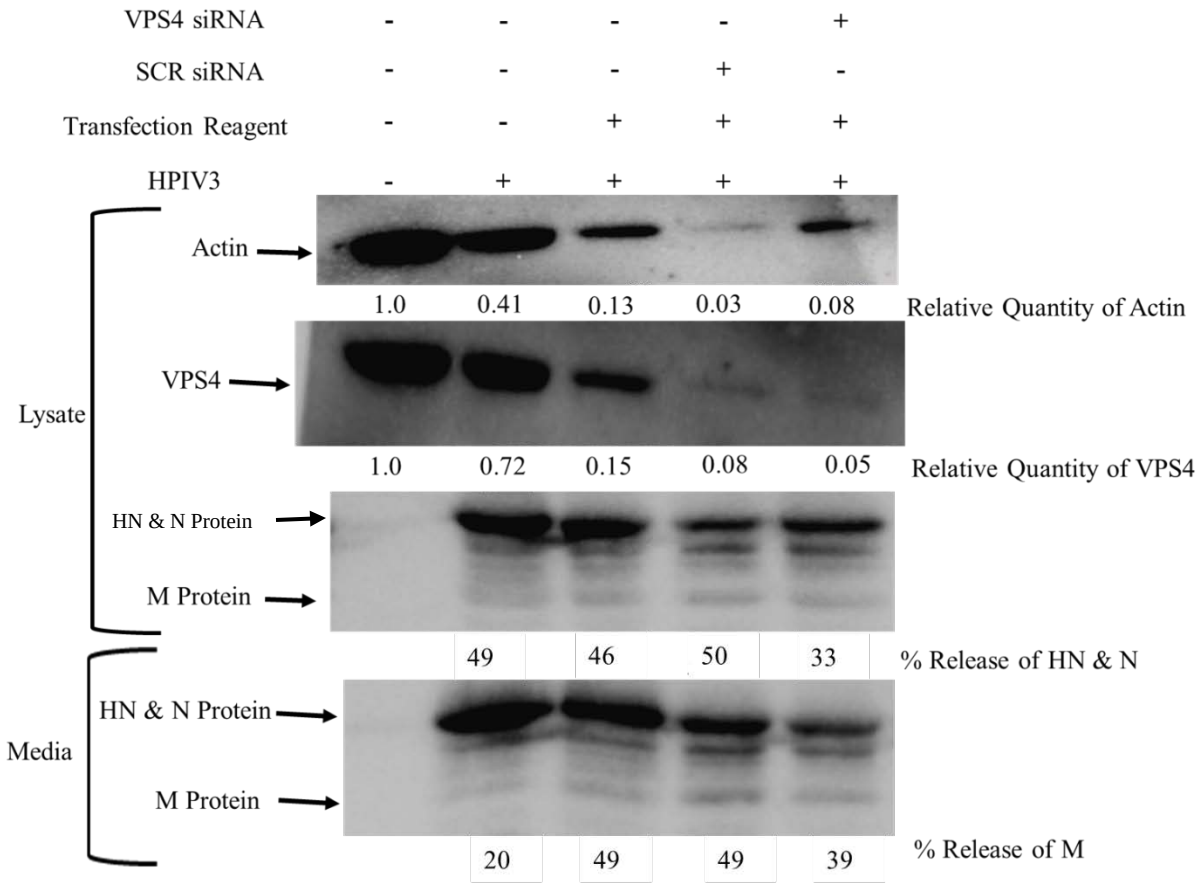

FIG. 11. Effect of VPS4A/B knockdown in LLC cells on HPIV-3 viral release. LLC cells were transfected with siRNA and 24 hours post transfection were transfected again with siRNA and infected with HPIV-3. At 72 hours post initial transfection media and cell lysates were harvested, and the media was subjected to ultracentrifugation through 20% sucrose. Proteins from both the media and lysates were separated via SDS-PAGE, and western blotted.

Upon further analysis, when multiple experiments were averaged, it was observed that there was a mean VPS4 decrease of 90% in the siRNA + Transfection reagent lysates (column 5) compared to the Mock lysates (column 1) (Fig. 12A). However, comparison of the Transfection Reagent only lysates (column 3) to the siRNA + Transfection reagent lysates (column 5) showed a 50% decrease in VPS4 (Fig. 12A). When the budding efficiencies of multiple experiments were viewed, a 10% decrease in the budding of M and a 14% decrease in the budding of HN + N (FIG 12B) were observed when comparing

the Transfection Reagent only lysates (column 3) to the siRNA + Transfection Reagent lysates (column 5). However, it should be noted that release of M protein with VPS4-specific siRNA is still above levels seen in a natural infection without transfection reagent present (Column 1, FIG 12B).

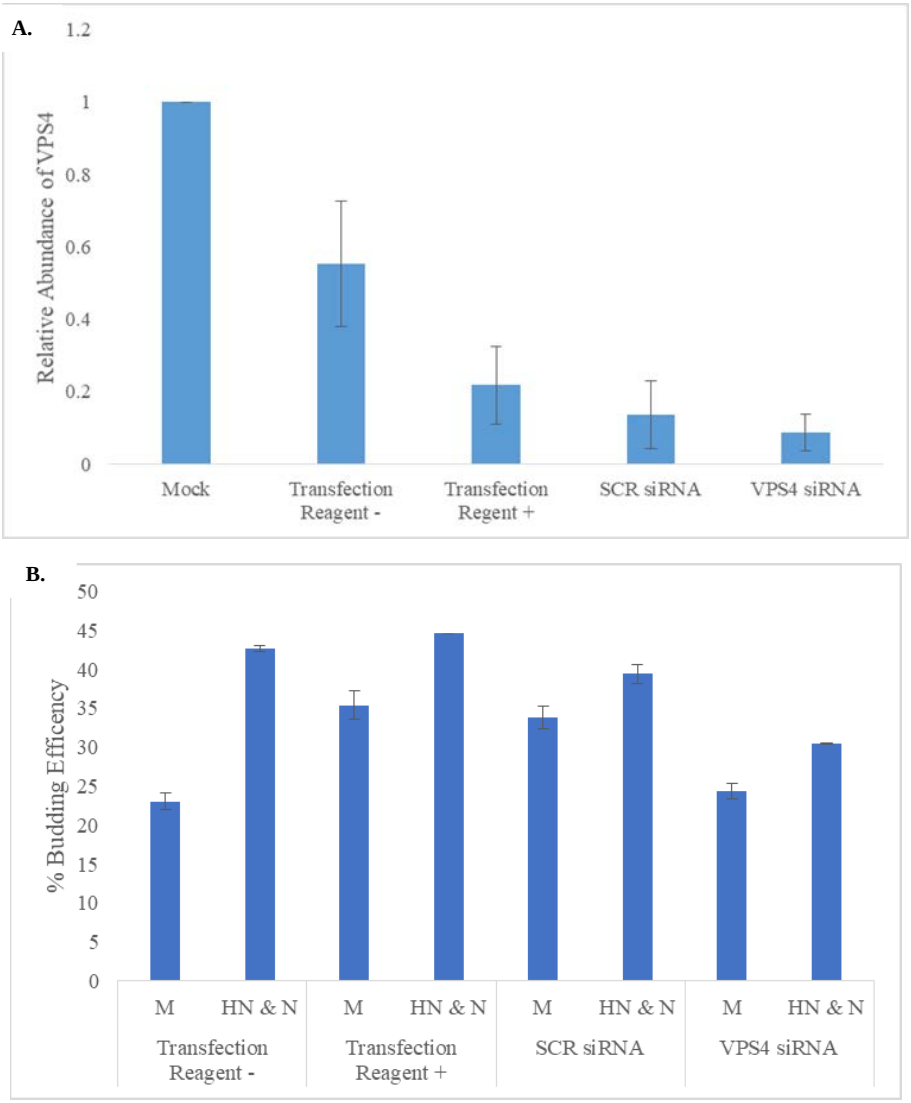

FIG. 12. Average VPS4 expression and viral protein release. A. The average relative quantity of VPS4 compared to the uninfected mock lane. Standard deviations were calculated from four independent experiments. B. The average percentage of matrix, hemagglutinin and neuraminidase proteins released after the addition of siRNA and transfection reagent. Standard deviations were calculated from four independent experiments matrix and from two independent experiments for HN & N.

DISCUSSION

Enveloped viruses rely on their host's cellular proteins to carry out key steps of the viral life cycle. Here, we investigated the role of the ESCRT pathway and, more specifically, the necessity of VPS4 in the release of HPIV3 VLPs and particles.

To investigate the role of the ESCRT pathway in HPIV3 budding and M VLP formation, the first objective was knocking down VPS4, a protein involved in the final steps of the ESCRT pathway and critical for recycling of ESCRT components. Once VPS4 was sufficiently knocked down in cells, the effect on HPIV3 VLP and virus release was studied. There was a 50% decrease in the budding efficiency of HPIV3 VLPs with 99% VPS4 knockdown. Thus with substantial VLP release occurring under such VPS4 knockdown conditions, it leads to the conclusion that the ESCRT pathway is not critical for HIPV3 M-directed VLP release. During infection studies, the release of HPIV3 virus proteins was reduced by 10% when VPS4 was knocked down by 67%. The data indicates that the ESCRT pathway is not necessary for the release of HPIV3 VLPs and is inconclusive with respect to the necessity of the ESCRT pathway in virion release, due to experimental issues detecting proteins via western blots, with reproducibility.

This VLP data is in agreement with previous studies done looking at the involvement of ALIX, an accessory protein in the ESCRT pathway. Previous work in our lab failed to show interactions between ALIX and HPIV3 M, via co-immunoprecipitation and mammalian two-hybrid assays. Another study done by Suresh

Kandel involved the creation of stable cells lines via integration of plasmid DNA encoding siRNAs targeting ALIX mRNA into the genome of the cells. There was not any significant decrease in VLP release from ALIX-depleted cells (unpublished data by Suresh Kandel). This combined data is also in agreement with data from Gosselin-Gernet et al. wherein they showed no involvement of ALIX and VPS4 in SeV virus production. As mentioned in the Introduction, Gosselin-Gernet et al. depleted ALIX using siRNA while VPS4 was inhibited using dominant negative forms (84). However, our results and those of Gosselin-Gernet are in discordance with Irie et al., in which siRNA-mediated knockdown of ALIX was observed to significantly reduce SeV VLP release (53). The work done on SeV has been contradictory amongst itself in whether the ESCRT pathway is necessary for viral release, making it difficult to draw a conclusion.

There are other viruses that have shown a negative effect on budding after VPS4 knockdown. Schmitt et al., found that expression of VPS4 dominant negatives blocked budding of SV5 (52), while Kieffer et al., found knocking down VPS4 using siRNA duplexes caused a decrease in HIV-1 Gag budding (87). Both Schmitt et al. and Kieffer et al. showed similar knockdown of VPS4 to what our lab attained in this experiment giving stronger support to the claim that ESCRT is not likely to be utilized for HPIV3 VLP budding (52, 87). The total knockdown of VPS4 seen from the above data in LLC MK2 and 293T cells ranged from 99%-88%. The remaining amount of VPS4 expression is a potential draw back to this methodology, as these low levels of VPS4 expression could be sufficient for assisting HPIV3 budding. Although this minimal amount of VPS4 did not appear to be sufficient enough to facilitate Gag budding in HIV-1 when expressed in Helas (87).

The methodology used for conducting these knockdown style experiments could be a reason for the differences seen amongst research groups. Most methodologies only either use siRNA or dominant negative knockdown systems, one possible alternative approach could be combing both siRNA duplexes and dominant negatives, to decrease functional VPS4 even further. Although this extreme of a knockdown could result in unviable cells due to the normal function of VPS4 in cellular division. Also it may be beneficial to try other cells lines, our lab experienced issues getting consistent virus particle release from LLC MK2 cells, so a different cell line might give more consistent results for virus release. Also certain cell lines may be more sensitive to transfection reagents than others, giving varying results. Our lab also noticed that there was a decrease in cells when transfection reagents were added indicating that the reagents were harsh on the cells which could affect the amount of viable cells left for protein production. This could have been a reason for the lack of protein detection in the HPIV3 viral infection experiments.

The HPIV3 viral infection experiments experienced an array of problems one way to further investigate some of the values that were seen in the above data would be to try and see how much of the virus is viable and infectious. One plaque assay was attempted, using dilutions of the media aspirate from one of the viral infection experiments but no results were obtained. In turn one way to try and see if viable virus levels are reduced in tissue culture cells, while VPS4 is knocked down would be to preform plaque assay experiments. This would allow for being able to titer the amount of virus that is produced under each condition and whether or not viable virus particles are still being produced. In addition it would also assist in being able to determine if the protein levels

that were seen in the data above were from viable virus or from proteins released from apoptotic host cells, giving a better indication if the lack of VPS4 affects viral budding and release.

Another reason for the discrepancies that were seen between the two experiments presented along with the ones seen between SeV research groups could be due to the fact that when whole virus is present there are more protein interactions that occur that are not present when only the M protein is present in cells. One way particles could be formed was demonstrated with NDV where membrane deformation and vesicle budding have been shown in vitro using purified M protein and unilamellar vesicles, showing that the M protein is able to induce curvature and fission of the membrane alone, with no other accessory proteins (88, 89). This curvature is thought to be caused by M-M interactions forming a lattice structure on cellular membranes (88). This leads to the thought that the interactions between M proteins could be enough to promote budding on their own, without the involvement of cellular proteins. It is still unclear as to how HPV3 releases from cells without the use of the ESCRT proteins, but with further investigation into other cellular proteins and M-M interactions a clearer picture may become apparent.

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