HUMAN CYTOMEGALOVIRUS UTILIZES ESCRT-III AND EXTRACELLULAR VESICLES TO ENHANCE VIRUS SPREAD

A Dissertation in
Biomedical Sciences
by
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Abstract

Human cytomegalovirus (HCMV) is a highly prevalent human pathogen that can cause disease in the developing fetus and immunocompromised patients. A better understanding of the replication cycle of the virus may lead to better therapeutic targets to help control infection. During HCMV infection, cellular membrane from vesicles and organelles localize to a perinuclear region within the cell, which is termed the cytoplasmic viral assembly compartment (cVAC). This is the site of envelopment of progeny virions. Both viral and cellular proteins have been suggested to be involved during this process, but the mechanism of HCMV envelopment within the cVAC remains unknown. Many enveloped viruses utilize the endosomal sorting complexes required for transport (ESCRT) proteins for viral budding and envelopment. The ESCRTs are required for the membrane scission event during intraluminal vesicle (ILV) formation. The late ESCRTs, ESCRT-III and vacuolar sorting-associated protein 4 (VPS4), are responsible for membrane constriction and vesicle formation. Previous data suggest a potential role for the late ESCRTs during HCMV infection, but it is unclear whether the ESCRTs promote HCMV envelopment or a different stage of the replication cycle.

We hypothesized that ESCRT-III and VPS4 are required for HCMV envelopment at the cVAC. The role of the late ESCRTs was investigated through the inducible expression of dominant negative ESCRT subunits to inhibit ESCRT activity at various stages of HCMV infection. We found that ESCRT-III was not required for final envelopment of HCMV, as enveloped intracellular and extracellular virions could be found in infected cells when ESCRT activity was blocked. However, expression of
dominant negative ESCRT-III subunits slowed HCMV spread during a low multiplicity of infection (MOI).

Cells release signaling molecules inside membrane-enclosed compartments termed extracellular vesicles (EVs) that transfer proteins and nucleic acids to recipient cells. Virus infection commonly alters this signaling pathway by affecting the signaling molecules loaded as cargo into EVs. Changes in EV composition will impact whether they inhibit virus infection or promote an environment more conducive to replication and spread. EV biogenesis and release can occur through a mechanism that involves the ESCRT machinery. We hypothesized that inhibition of ESCRT activity may slow HCMV spread by preventing the release of proviral EVs that enhance virus spread to uninfected cells.

We found that HCMV upregulates ESCRT protein expression during infection, which coincides with increased EV biogenesis. Purification of EVs from HCMV-infected fibroblasts identified that infection increased vesicle release and altered vesicle size compared to EVs from uninfected cells. EVs purified from infected cells also contained viral factors known to be important for the initiation of the early stages of infection. The importance of EVs on HCMV spread was confirmed as treatment with the EV inhibitor GW4869 also slowed virus spread, and the transfer of EVs purified from HCMV-infected cells increased the efficiency of HCMV spread. Together, this work shows that HCMV modulates the EV pathway to transfer proviral signals to uninfected cells that prime the cellular environment for incoming infection.
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<td>ALIX</td>
<td>ALG-2 interacting protein X</td>
</tr>
<tr>
<td>BAR</td>
<td>Bin1/amphiphysin/RVs167</td>
</tr>
<tr>
<td>Cep55</td>
<td>centrosomal protein 55</td>
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<tr>
<td>CHMP</td>
<td>charged multivesicular body protein</td>
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<tr>
<td>COVID-19</td>
<td>coronavirus disease 2019</td>
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<tr>
<td>CPE</td>
<td>cytopathic effect</td>
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<tr>
<td>CRISPR</td>
<td>clustered regularly interspaced palindromic repeats</td>
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<tr>
<td>cVAC</td>
<td>cytoplasmic viral assembly compartment</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dubelcco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>dpi</td>
<td>days post infection</td>
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<tr>
<td>EAP</td>
<td>ELL-associated protein</td>
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<td>EBV</td>
<td>Epstein-Barr virus</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
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<tr>
<td>EM</td>
<td>electron microscopy</td>
</tr>
<tr>
<td>ESCRT</td>
<td>endosomal sorting complex required for transport</td>
</tr>
<tr>
<td>EV</td>
<td>extracellular vesicle</td>
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<tr>
<td>EV71</td>
<td>enterovirus 71</td>
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<tr>
<td>g</td>
<td>glycoprotein</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<td>GTP</td>
<td>guanosine triphosphate</td>
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<td>Description</td>
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<tr>
<td>GUV</td>
<td>giant unilamellar vesicle</td>
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<td>hepatitis A virus</td>
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<td>HCMV</td>
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<td>hepatitis C virus</td>
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<td>human dermal fibroblasts</td>
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<td>human embryonic kidney cell line</td>
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<td>human herpesvirus</td>
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<td>human immunodeficiency virus</td>
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<tr>
<td>hnRNPA2B1</td>
<td>heterogeneous ribonucleoprotein A2B1</td>
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<tr>
<td>hpi</td>
<td>hours post infection</td>
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<tr>
<td>Hrs</td>
<td>hepatocyte growth factor-regulated tyrosine kinase substrate</td>
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<td>HSPG</td>
<td>heparin sulfate proteoglycan</td>
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<td>HSV</td>
<td>herpes simplex virus</td>
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<tr>
<td>IE</td>
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<tr>
<td>ILV</td>
<td>intraluminal vesicle</td>
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<td>JCPyV</td>
<td>JC polyomavirus</td>
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<td>KSHV</td>
<td>Kaposi’s sarcoma-associated herpesvirus</td>
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<tr>
<td>IncRNA</td>
<td>long noncoding RNA</td>
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<td>major histocompatibility complex</td>
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<td>major immediate-early promotor</td>
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<td>MIM</td>
<td>MIT interacting motif</td>
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<tr>
<td>miRNA</td>
<td>microRNA</td>
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<tr>
<td>MIT</td>
<td>microtubule interacting and transport</td>
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<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<td>MTOC</td>
<td>microtubule organizing center</td>
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<tr>
<td>MVB</td>
<td>multivesicular body</td>
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<tr>
<td>NEC</td>
<td>nuclear egress complex</td>
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<td>nSMase</td>
<td>neutral sphingomyelinase</td>
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<td>OR14I1</td>
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<td>PDGFRα</td>
<td>platelet-derived growth factor receptor alpha</td>
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<td>phosphoprotein</td>
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<tr>
<td>PtdIns3p</td>
<td>phosphatidylinositol 3-phosphate</td>
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<td>RNAi</td>
<td>RNA interference</td>
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<td>RSV</td>
<td>Rous sarcoma virus</td>
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<tr>
<td>SARS-CoV-2</td>
<td>severe acute respiratory syndrome-related coronavirus 2</td>
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<tr>
<td>SYNCRIP</td>
<td>synaptotagmin binding cytoplasmic RNA interacting protein</td>
</tr>
<tr>
<td>TGN</td>
<td>trans-Golgi network</td>
</tr>
<tr>
<td>Tsg101</td>
<td>tumor susceptibility gene 101</td>
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<tr>
<td>UBL3</td>
<td>ubiquitin-like 3</td>
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<tr>
<td>UIM</td>
<td>ubiquitin interacting motif</td>
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<tr>
<td>UL</td>
<td>unique long</td>
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<tr>
<td>Vps or VPS</td>
<td>vacuolar protein sorting</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>Vta1</td>
<td>vesicle trafficking 1</td>
</tr>
<tr>
<td>VZV</td>
<td>varicella-zoster virus</td>
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<tr>
<td>YBx1</td>
<td>Y box binding protein 1</td>
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Preface

Chapter 2 and 3 of the dissertation include multiple-authored work. In chapter 2, Jillian Carmichael performed the experiments involving herpes simplex virus infection. This data is found in Figure 2-2E and Figure 2-6C. In chapter 3, Yuanjun Zhao and Jeffrey Sundstrom contributed to the data acquisition and analysis of purified extracellular vesicle populations by nanoparticle tracker analysis. This data is found in Figure 3-4C and Figure 3-4E.
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reason to smile.

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incredible training opportunity that also provided funding that was used for this work
through NIH training grant T32CA060396. The findings and conclusions presented in
this dissertation do not necessarily reflect the views of the funding agencies.
Chapter 1: Introduction
Herpesviruses are ubiquitous pathogens that establish life-long infections within their host. The herpesviruses are classified into subfamilies based on characteristics of their replication cycle and cell tropism. Human herpesviruses (HHV) can be found in all subfamilies. The alphaherpesvirus family includes herpes simplex virus type 1 and 2 (HSV-1, HSV-2 or HHV-1, HHV-2 respectively) and varicella zoster virus (VZV or HHV-3). The alphaherpesviruses are characterized by a relatively short replication cycle in cell culture and latent infection is associated with neuronal cells [1]. The betaherpesvirus family includes human cytomegalovirus (HCMV or HHV-5) and HHVs 6A, 6B, and 7. The betaherpesviruses have a prolonged replication cycle in cell culture and have a restricted host tropism [1]. The gammaherpesviruses are Epstein-Barr virus (EBV or HHV-4) and Kaposi’s sarcoma associated herpesvirus (KSHV or HHV-8). The gamma herpesviruses are associated with infection of cells from the lymphoid lineage, and KSHV and EBV are both associated with multiple types of cancer. However, not all of the associated cancers are of lymphoid origin [1].

**Human cytomegalovirus**

HCMV shares common structural components of the viral particle with the other herpesviruses (Figure 1-1). The outermost layer of the particle consists of a lipid membrane that surrounds the virion, which is referred to as the viral envelope. The envelope contains glycoprotein complexes that are important for attachment and entry into host cells. The envelope surrounds an icosahedral protein capsid that contains the viral genome.
Figure 1-1: Structural components of HCMV. A) Diagram of the components of the HCMV virion. B) Transmission electron micrograph of extracellular HCMV released from an infected fibroblast. Scale bar: 50 nm.
The layer between the envelope and the capsid consists of protein and nucleic acids that are packaged into the viral particle during the assembly process. This layer, called the viral tegument, contains factors that play important roles during the initiation of infection [2-4]. The double-stranded DNA genome is found inside the protein capsid. HCMV has the largest genome of the herpesviruses, which is approximately 230 kilobase pairs and encodes over 200 proteins and noncoding RNAs [5, 6]. The infectious particle is approximately 200 nanometers in diameter.

**HCMV clinical significance**

HCMV is a common pathogen, and seroprevalence can be as high as 70-90% in certain populations of the United States [7]. Infection is restricted to cells of human origin, but viruses with significant sequence homology have been found in other animals including rodents and nonhuman primates [8]. These viruses are important models of HCMV in vivo. The virus is commonly spread through bodily secretions and it is typically acquired early in life. The main source of transmission is thought to be young children, which have higher shedding rates of HCMV than other age groups [9]. Healthy individuals typically develop asymptomatic infections to HCMV, therefore it is not considered a major health concern to the general population. However, consequences of subclinical infections may not be fully appreciated. Primate models of subclinical infections have altered immune cell distribution, changes in the composition of gut microbiota, and reduced response to vaccination [10].

The most serious clinical consequences of HCMV infection occur in newborns and immunocompromised individuals. HCMV is the “C” in the T.O.R.C.H. acronym used to describe pathogens that are capable of causing infection in utero [11, 12].
Transmission rates from the pregnant mother to the fetus occur in approximately 1% of pregnancies, and pathology associated with infection can be severe. The most common pathologies in symptomatic newborns include hearing loss, vision impairment, and cognitive or motor defects [13, 14]. Transplant patients are also at risk of complications caused by HCMV infection. HCMV DNA and viral antigens are commonly detected in solid organ and hematopoietic transplant patients [15-17]. Complications can include systemic febrile illness (referred to as CMV syndrome), graft rejection, and organ dysfunction [8].

Antivirals are available to treat HCMV infection and prophylactic treatment regimens have reduced the burden of disease [18-21]. Two commonly used antivirals are ganciclovir and valganciclovir, which inhibit viral DNA synthesis. Letermovir was recently approved by the Federal Drug Administration for prophylactic treatment of hematopoietic cell transplant patients. Letermovir inhibits the terminase complex needed to package the DNA genome into the capsid. Antivirals represent important tools for the treatment of HCMV-mediated disease, but drug-resistant strains arise for all available treatment options [22-25]. Toxicity associated with certain antiviral therapies also limits the efficacy of the options that are currently available. Transplant patients receiving prophylactic treatment with certain approved therapeutics have reduced risk of HCMV disease, but the overall mortality rate was not improved [26]. In these studies, patients were protected from HCMV disease but the toxicity associated with the antivirals resulted in serious clinical complications independent of HCMV infection. The toxic effects of the antivirals are likely the reason that overall mortality rates did not decrease. This highlights the need for more therapeutic options to control infection in at-risk
patients. To discover new treatment options, it is necessary to gain a better understanding of the replication cycle of HCMV, which will be described in the following sections (Figure 1-2).

**HCMV entry**

HCMV can infect cells from the myeloid lineage, fibroblasts, epithelial, and endothelial cells. Myeloid progenitor cells are of particular importance, because these are the reservoir of HCMV latency in the host [27, 28]. HCMV encodes multiple proteins found on the viral envelope that are necessary for virus entry [29]. The first step during virus entry involves initial attachment to the host cell. Attachment to the cell allows interactions between viral proteins and the entry receptors to occur. The glycoproteins gM and gN interact with heparin sulfate proteoglycans (HSPGs) and gB interacts with integrins [30-32]. Preventing either of these interactions blocks attachment to the cell and the subsequent infection.

Following attachment, entry occurs through either macropinocytosis or endocytosis [33, 34]. The route of entry is dependent on the cell type and the presence of certain protein complexes on the surface of the viral envelope. The trimeric complex is made up of the viral glycoproteins gH, gL, and gO. The trimeric complex is required for all infections as viruses that do not contain the trimeric complex in the viral envelope cannot infect any cell type [35, 36]. The trimeric complex facilitates entry through interactions with platelet-derived growth factor receptor alpha (PDGFRα) [37-40]. The pentameric complex is made up of the viral proteins gH, gL, UL128, UL130, and UL131.
Figure 1-2: Stages of HCMV lytic replication.
This is required for infection of endothelial, epithelial, and myeloid cells, but not required for fibroblast infection [41, 42]. Pentameric complex-dependent entry into cells is through the endocytic route [33].

A screen of cellular membrane proteins identified that neuropilin-2 interacts with the pentameric complex and is important for epithelial and endothelial cell infection [43]. Additionally, a CRISPR screen identified that olfactory receptor 14I1 (OR14I1) is also important for entry into epithelial and endothelial cells [44]. HCMV entry in different contexts is likely dictated by numerous factors including the abundance of these receptors, and levels of the pentameric and trimeric complexes on the viral surface [45]. Regardless of the entry receptor used, all upstream interactions result in the activation of gB, the viral fusogen, which fuses cellular membrane with the viral envelope and allows the capsid to gain access to the cytoplasm [46, 47]. Once in the cytoplasm, capsids in the associate with microtubules, and traffic to the nucleus where they colocalize with markers of the nuclear pore complex [48]. Once the capsid has docked at the nuclear pore, the genome is inserted into the nucleus and viral gene expression is initiated.

**HCMV gene expression and genome replication**

Viral gene expression is temporally regulated during HCMV infection. Within the viral genome, each open reading frame is classified as an immediate-early (IE), early, or late gene based on expression kinetics during infection. IE expression occurs within hours after genome entry into the nucleus and can be detected through all stages of infection. Expression of the IE proteins occurs from the major immediate-early promoter (MIEP) [49-52]. HCMV requires the activity of viral proteins to initiate expression of the MIEP, which are packaged into the viral tegument. An essential tegument protein is the
UL82 gene product pp71, which acts as a transactivator for MIEP expression [53, 54]. Expression of the IE proteins is required to counteract cell-mediated transcriptional silencing of the viral genome, and to initiate expression of the HCMV early proteins [55-57].

HCMV replication and assembly impose huge demands on the resources of the infected cell. A large number of viral factors expressed with early kinetics establish an environment where HCMV replication and assembly can occur efficiently. Cellular metabolic activity is significantly altered to support the energetic demands of infection. Early genes expression can be detected between 24 and 48 hours post infection and encode proteins that cause an increase in glucose uptake, activate pyrimidine synthesis, and remodel lipid metabolism to meet the increased need for lipid synthesis during infection [58-60]. HCMV early genes also alter the cell cycle to prevent cellular DNA replication and ensure that factors necessary for DNA synthesis available for replication of the viral genome [61]. Proteins and miRNAs are involved in promoting a G0/G1 cell cycle arrest [62, 63]. Viral proteins and miRNAs expressed with early kinetics cause this arrest through extensive targeting of cyclin activity [64-67]. Early proteins also prevent the activation of the innate immune response. This is through interactions with cytosolic DNA sensors and inhibition of antiviral signaling pathways [67-73]. Finally, viral proteins are primarily responsible for genome replication, and they are expressed with early expression kinetics. DNA synthesis is carried out through rolling circle replication by UL54, the viral polymerase, and the processivity factor UL44 [74-77]. The viral helicase/primase complex and DNA binding proteins also contribute to replication of the genome [78-80].
The final set of viral ORFs to be expressed are the late genes. To be classified as a late gene, expression of the ORF must be dependent on replication of the viral genome. This is confirmed experimentally by showing that expression of late genes is blocked by inhibitors of viral DNA replication [8]. Late genes encode the structural proteins necessary to generate the infectious particle and their expression can be detected by 72 hours post infection. After late gene expression, capsid assembly and genome packaging initiate the viral assembly process.

**HCMV assembly and envelopment**

Following genome packaging in the nucleus, the capsids must traffic through the nuclear envelope to gain access to the cytoplasm to complete viral assembly. Viral and cellular proteins form a nuclear egress complex (NEC) to disrupt the nuclear lamina and expose capsids to the nuclear membrane [81, 82]. Electron microscopy analysis of nuclear egress identified that capsids bud into the perinuclear space and undergo a de-envelopment step to gain access to the cytoplasm [83]. While the components of the NEC are well defined, the cellular and viral proteins involved in the budding event during nuclear egress are still unknown. Next, capsids enter the cytoplasmic viral assembly compartment (cVAC). The cVAC is an accumulation of membrane from vesicles and organelles that are reorganized by viral and cellular factors during infection [84-87]. A microtubule organizing center (MTOC) derived from the Golgi apparatus is found at the center of the cVAC, and the MTOC is responsible for increased accumulation of membrane adjacent to the nucleus [88]. Immunofluorescence analysis using markers of different vesicles and organelles suggests that cVACs form in an ordered structure, as membrane markers localize to specific regions within the cVAC (Figure 1-3) [89, 90].
Figure 1-3: Membrane reorganization during HCMV cVAC formation. Model of membrane organization within the HCMV cVAC based on the origin of cellular membrane markers. MT: microtubule, MTOC: microtubule organizing center.
In a largely undefined process, capsids acquire tegument proteins and undergo viral envelopment during the final stages of assembly in the cVAC [91-93].

Envelopment in the cVAC is the final stage during the production of infectious HCMV particles. While numerous studies have used electron microscopy to define the cVAC as the site of envelopment, distinct details of this event are still limited. For example, the origin of the membrane used for HCMV envelopment is still not well defined. Understanding the origin of the viral envelope may help determine what other factors are present at the site of envelopment. For example, the membrane used for envelopment of HSV-1, a member of the alphaherpesvirus family, is membrane tubules associated with the endocytic membrane recycling pathway [94]. Identifying viral and cellular proteins that localize to this membrane can provide important information about the factors required for HSV-1 envelopment. The typical organelle morphology and organization is disrupted to generate the cVAC during HCMV infection, which makes this more difficult to identify the source of membrane using traditional microscopy methods. Electron microscopy analysis of different zones of the cVAC has identified that viral envelopment occurs throughout the compartment without any spatial bias [95]. In agreement with this, studies have identified that the viral envelope contains markers associated with various vesicle types and organelles [93, 96, 97]. This data can be explained by two different hypotheses. The first hypothesis is that HCMV envelopment can occur at different membrane sources during infection. The second hypothesis is that membrane mixing occurs during cVAC formation, which prevents the use of traditional membrane markers to identify the origin of the envelope. These two hypotheses cannot
be distinguished by studying a population of viral particles, but instead must be addressed through single particle analysis and lipidomic profiling.

In addition to a limited understanding of the source of the membrane used, a clear definition of the viral and cellular proteins involved in envelopment is also lacking. HCMV envelopment can be broken down into two stages: (i) recruitment of capsids to sites of envelopment followed by initial membrane wrapping of the capsids, and (ii) the membrane scission event needed to fully enclose the capsid inside an envelope. Numerous viral proteins found in the tegument (pp28, UL94, UL47, and UL97) and lipid envelope (gN and gO) are required to recruit capsids to sites of envelopment [98-104]. Deletion of any of these proteins results in the accumulation of naked capsids in the cytoplasm. It is difficult to determine the individual role of any of these proteins because of the complex network of interactions among tegument proteins. Disruption of any interaction important for proper assembly of the tegument layer will likely disrupt subsequent capsid recruitment to the membrane. The inability to dissect tegument formation and membrane targeting has complicated interpretations about viral proteins that are required to recruit capsids to membranes to initiate envelopment.

The second stage of envelopment is the membrane scission event. Currently, there are only two viral proteins directly implicated in membrane scission during viral envelopment. Viruses null for the expression of UL71 or UL103 have reduced infectious virus production [87, 105, 106]. This arrest appears to be at a very late stage, leaving virus buds still attached to the host membrane [106]. This indicates a defect in membrane scission needed to generate the infectious particle. Further analysis of UL71 has identified key domains necessary for envelopment, including a YXXΦ motif necessary
for membrane targeting and trafficking as well as a leucine zipper motif required for protein oligomerization [107-109]. UL71 is clearly involved during the late stages of membrane scission, but the mechanism is unclear. Is UL71 responsible for membrane scission, or is UL71 important for the recruitment of other proteins known to promote the scission of membrane? The experiments in this dissertation will investigate the hypothesis that cellular proteins known to mediate membrane scission events function with UL71 to mediate HCMV envelopment. A detailed description of the candidate cellular proteins is provided in the next section.

**HCMV egress and spread**

Following envelopment, HCMV must facilitate spread of the virus to new uninfected cells. Since envelopment does not occur at the plasma membrane, the infectious particle must be transported to the periphery of the infected cell and released, a process called virus egress. Transcriptomic changes during HCMV infection show that HCMV dramatically alters expression of proteins involved in cellular vesicle trafficking and secretion [110]. In addition to this, a number of cellular proteins involved in vesicle trafficking were found to be important during HCMV infection in a large siRNA screen [111]. These studies suggest that HCMV utilizes cellular machinery involved in vesicle trafficking and exocytosis to facilitate virus egress. This was supported by a study showing that knockdown of Rab27, a GTPase involved in vesicular trafficking and secretion, reduced the release of virus from infected cells [112]. HCMV appears to utilize cellular secretion pathways to facilitate egress, but the viral and cellular interactions required for this event and whether the same pathways are used in all cell types are still under investigation.
Once HCMV is transported to the cellular periphery, the virus can spread to a new cell through two distinct mechanisms. One mechanism is referred to as cell-to-cell spread. During infection, especially with clinical isolates of HCMV, relatively small amounts of infectious virions are released into the extracellular environment [113-115]. Within a host, the majority of virus spread is thought to occur through direct cell-to-cell contact, where virus is passed through junctions formed between cells [116]. In support of this, HCMV spread in cell culture still occurs in the presence of neutralizing antibody or viscous media overlays that do not allow the transfer of extracellular molecules to surrounding cells [117, 118]. This mechanism of cell-to-cell spread is still dependent upon viral glycoproteins, suggesting some requirement for the entry machinery [115].

HCMV that is released into the extracellular environment can infect new cells through cell free spread. Cell free spread, which is likely important for dissemination throughout a host and transmission to new hosts, is sensitive to neutralizing antibodies. In addition to infectious virus, other membrane enclosed particles are also released from infected cells. Cells release particles that contain viral proteins, mRNAs, and miRNAs but lack a capsid or genome [4, 119]. These particles are referred to as dense bodies, and it is not clear what effect dense bodies have on cells that internalize them [120]. However, they appear to have an important function in immune activation. Dense bodies contain the surface glycoproteins present on the viral envelope. The dense bodies are highly immunogenic, and produce a neutralizing antibody response to the glycoproteins when transferred into animal models [121]. One theory is that dense bodies may serve as an antibody decoy that interacts with antibodies and increases the chance that cell free virus is not neutralized.
In addition to neutralizing antibodies, HCMV must also overcome other extracellular factors to facilitate efficient spread of the virus. This can be accomplished through quite different mechanisms. Bodily fluids, such as seminal plasma, contain inhibitory factors that block HCMV entry [122]. Cell-to-cell communication can also activate signaling that contributes to the control of virus infection. Co-culture of monocyte-derived cells with infected epithelial cells reduced virus spread in epithelial cells [123]. In these experiments the transfer of supernatant from the monocyte-derived cells to epithelial cells was sufficient to slow virus spread, suggesting that secreted factors were responsible for controlling infection. To efficiently spread, HCMV must have ways to block or regulate these intracellular signaling mechanisms. How HCMV regulates these pathways is largely unknown.

The ESCRT Machinery

Membrane scission

Membrane scission proteins are required for a wide range of cellular processes. Cells require a diverse set of proteins involved in membrane scission to accommodate differences in the membrane topology of these scission events. Membrane scission is required to generate vesicles budding into or away from the cytoplasm (Figure 1-4A). During receptor-mediated endocytosis, the newly forming vesicle buds into the cytoplasm. Cytoplasmic coat proteins, such as clathrin and coat protein (COP) I and II complexes, can act on the outer surface of the vesicle to induce membrane curvature and provide the energetic force needed to scission membrane.
Figure 1-4: Membrane scission by the ESCRT machinery. A) Topology of membrane budding events within cells. Receptor-mediated endocytosis generates vesicles that bud toward the cytoplasm, which requires membrane scission machinery to act on the outside of the bud neck. Intraluminal vesicle formation and vesicle budding at the plasma membrane require the ESCRT machinery for membrane scission inside the bud neck. B) Schematic of ESCRT-mediated degradation of membrane proteins through MVB formation.
Membrane-binding proteins that contain Bin/amphiphysin/Rvs (BAR) domains are known to be important for membrane deformation as part of this process [124-127]. These membrane remodeling proteins also recruit proteins such as dynamin that provide the force needed to constrict and scission membrane to generate a new vesicle [128, 129]. Dynamin forms oligomeric rings on the outside of the bud neck and constricts the membrane through conformational changes that are dependent on the hydrolysis of guanosine triphosphate (GTP) [130-134]. This conformational change is thought to provide the force necessary for scission to form the new endocytic vesicle.

In the scenario described above, membrane curvature and scission occur through the activity of cytoplasmic proteins present on the outer surface of the newly forming vesicle, but cells must also have the ability to scission membrane from the inner surface of newly forming vesicles as well. Following endocytosis, membrane proteins on the endocytic vesicle can be degraded by the lysosome. Fusion of endosomal vesicles with the lysosome results in the degradation of internalized cargo. However, membrane proteins cannot be fully degraded through fusion alone, because the cytoplasmic portion of the protein will remain intact and protected from the lysosomal enzymes (Figure 1-4B). For this reason, membrane proteins must be internalized into invaginations of the endosomal membrane to make intraluminal vesicles within the endosome [135-137]. Endosomes containing ILVs are referred to as multivesicular bodies (MVBs). In events such as MVB formation, proteins must generate membrane curvature and scission of vesicles budding away from the cytoplasm.

Membrane scission of vesicles forming away from the cytoplasm is driven by a set of protein complexes termed the endosomal sorting complexes required for
transport (ESCRT). The ESCRT proteins were first identified in a screen for genes responsible for the proper sorting of membrane proteins to the yeast vacuole [138]. Out of all identified genes, a subset of mutants were found to prevent the targeting of membrane proteins to the vacuole and these mutants caused membrane proteins to accumulate at a novel compartment referred to as a class E compartment [139]. Class E mutants no longer internalized membrane proteins into the lumen of endocytic compartments, suggesting that these proteins are responsible for the formation of ILVs [140]. Genes associated with the class E phenotype were eventually characterized as members of the ESCRT machinery.

**ESCRT complexes and their function**

The initial work to determine the composition and function of the ESCRT proteins was carried out in yeast, and later work identified mammalian orthologs that have similar functions for degradation in the lysosome [141]. For clarity, only mammalian names of the ESCRT subunits will be used in the text, but a list of the subunits of the core ESCRT machinery and their yeast orthologs are listed in Table 1-1. The ESCRTs include five protein complexes termed ESCRT-0, I, II, III, and VPS4. The complexes are thought to function sequentially during ILV formation, and they have been numerically ordered according to this model. Biochemical analysis of the ESCRTs led to the identification of the protein subunits that make up each complex.

ESCRT-0 originally consisted of hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) and signal transducing adapter molecule 1/2 (STAM 1/2) [142, 143]. However, a growing list of ESCRT-0 adaptor proteins continues to be identified.
<table>
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<th>Complex</th>
<th>Mammalian Subunit</th>
<th>Yeast Subunit</th>
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<td>Vps27</td>
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<td></td>
<td>STAM 1/2</td>
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<td>ESCRT-I</td>
<td>Tsg101</td>
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<td>hVps28</td>
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<td>Vps37 (A, B, and C)</td>
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<td>hMvb12 (A and B)</td>
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<td>ESCRT-II</td>
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<td>ESCRT-III</td>
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<td>VPS4</td>
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* isoforms of mammalian subunits are listed in parentheses
ESCRT-I consists of four protein subunits: tumor susceptibility gene 101 (Tsg101), human vacuolar protein sorting-associated protein (hVps28), Vps37 (isoforms A, B, and C), and human multivesicular body sorting factor 12 (hMvb12) (isoforms A and B) [144-151]. ESCRT-II is made up of three protein subunits. Each complex contains two molecules of ELL-associated protein 20 (EAP20) and one molecule of EAP30 and EAP45 [152-155]. Both ESCRT-I and ESCRT-II are found in stable complexes within the cytoplasm. In contrast, ESCRT-III subunits are found only in monomers in the cytoplasm and do not assemble into an oligomeric complex until recruited to membrane. Subunits of ESCRT-III include charged multivesicular body protein 6 (CHMP6), CHMP4 (isoforms A, B, and C), CHMP2 (isoforms A and B), and CHMP3 [156, 157]. The final complex of this pathway is VPS4. Mammalian VPS4 has two isoforms (A and B) and oligomerizes into a double ring structure made up of 12 VPS4 subunits when bound to ATP [158-161]. Two accessory factors, vesicle trafficking 1 (Vta1) and CHMP1, associate with the VPS4 complex and are known to increase the activity of VPS4 [162-164].

The ESCRTs drive the scission event during the budding of vesicles away from the cytoplasm. The first example of such an event was ILV formation, but the ESCRTs are now known to be required for many other cellular events that require this type of scission topology. The ESCRTs are recruited to sites of membrane damage at the nuclear envelope and plasma membrane, where they seal the ruptured membrane through membrane scission [165-167]. In these roles, the activity of the ESCRT machinery is essential to prevent cell death associated with the damage to the cell. The ESCRT machinery is also required during cytokinesis. During the final stages of cytokinesis, the
membrane and microtubule bundle between the two daughter cells must be severed. Without the activity of the ESCRTs, cell abscission cannot be completed, indicating that the ESCRTs are required by any dividing cell [168]. While the number of events known to require the ESCRT machinery may still increase, the currently defined roles of the ESCRTs all result in membrane deformation and scission.

The different complexes of the ESCRT machinery have unique functions during the membrane scission process (Figure 1-5). The activity of each complex allows for their categorization as early or late-acting ESCRTs. The early ESCRTs, ESCRT-0, I, and II, are important for their ability to interact with ubiquitinated protein cargo at membrane and for the ability to recruit the other ESCRT machinery to these sites. The Hrs subunit of ESCRT-0 contains a FYVE domain that interacts with phosphatidylinositol 3-phosphate (PI3P) and is responsible for targeting the complex to endosomal membrane, which is enriched with PI3P [169, 170]. Hrs also interacts with ubiquitinated proteins through a ubiquitin interacting motif (UIM), which contributes to the targeting of ESCRT-0 to proteins on the endosomal membrane marked for lysosomal degradation [171]. The ESCRT-I subunit Tsg101 also contains protein domains that allow the protein to interact with ubiquitinated substrates [145]. Similarly, the EAP45 subunit of ESCRT-II contains a GLUE domain capable of interacting with PI3P and ubiquitin, and point mutations within this domain abolish the sorting and degradation of membrane proteins [172, 173]. The combination of these interactions results in a clustering of ESCRT-0, I, and II at sites on endosomal membrane that contain ubiquitinated cargo destined for lysosomal degradation. Next, the late-acting ESCRTs are recruited to facilitate progression of the membrane scission process.
Figure 1-5: Model of ESCRT recruitment and membrane scission. Early-acting ESCRTs (ESCRT 0, I, and II) are recruited to membrane through interactions with ubiquitin and lipid moieties. Late-acting ESCRTs are recruited to drive membrane constriction and scission.
Once early ESCRTs accumulate at membrane, they can then recruit subunits of ESCRT-III. The ESCRT-II subunit EAP20 interacts with CHMP6, which recruits this ESCRT-III subunit to the membrane [174, 175]. The recruitment of CHMP6 to the membrane causes nucleation of CHMP4 subunits and oligomerization at the membrane, followed by the recruitment of CHMP2 and CHMP3 [176, 177]. Alpha helices at the N-terminus of the ESCRT-III subunits contribute to membrane binding, and the formation of filaments on membrane [178, 179]. Oligomerization causes constriction of the membrane at the neck of the newly budding vesicle. There are several models to explain how ESCRT-III oligomers drive membrane scission, but the consensus is that constriction of membrane through the formation of spiraling ESCRT-III filaments ultimately brings the opposite sides of the bud neck in close enough proximity to fuse both sides of the bud neck and generate the vesicle [180-183].

The final complex recruited to membrane scission sites is VPS4. VPS4 is recruited by ESCRT-III subunits through interactions between the microtubule interacting and transport (MIT) domain on VPS4 and the MIT-interacting motif (MIM) domains on ESCRT-III subunits [184-186]. While all ESCRT-III subunits contain MIMs at their C-terminus, they do not contain the same amino acid sequence or functional relevance. CHMP6 and CHMP4 contain MIM1 sequences, while CHMP2 and CHMP3 subunits contain MIM2 sequences. MIM2 sequences appear to be more biologically relevant for VPS4 recruitment as only mutation of MIM2 sequences block VPS4 recruitment within cells [187]. VPS4 is clearly important for recycling ESCRT subunits from the membrane, but the precise role of VPS4 during membrane scission is still under investigation. VPS4 inhibition through loss of function mutations disrupts cargo
trafficking and degradation [158, 159]. Numerous studies suggest that the VPS4 ring structure and ATPase activity is required to disassemble ESCRT-III filaments at the sites of membrane scission [162-164, 180, 188, 189]. In vitro analysis of ESCRT-mediated scission on giant unilamellar vesicles (GUVs) suggests that the disassembly of ESCRT-III filaments from the membrane may generate the force needed to scission membrane [190]. However, other in vitro data using GUVs suggests that ESCRT-III oligomerization alone is required for the initial membrane scission event, and VPS4 is only necessary for recycling ESCRT-III subunits from their membrane-bound state for subsequent rounds of scission [180]. VPS4 is clearly required for the proper function of the ESCRTs in vivo, suggesting that ESCRT-III recycling from the membrane is an essential part of this process.

The sequential recruitment of the ESCRTs is a model established through experiments analyzing the recognition and trafficking of membrane proteins targeted for degradation. However, advances in microscopy techniques have allowed the study of recruitment kinetics of the ESCRT machinery at high resolution in live cells. These techniques have generated data that suggest the ESCRT machinery may not be recruited with sequential kinetics [191]. Further analysis will expand our understanding of the recruitment and activity of the ESCRTs during membrane scission. In addition to these new findings, alternative functions of the ESCRT machinery such as abscission during cytokinesis and membrane repair only utilize subsets of the ESCRT machinery. The early ESCRTs preferentially bind to endosomal membrane through ubiquitin interactions and protein domains that recognize lipids that are enriched on the surface of endosomes. Abscission and membrane repair do not occur at endosomal membranes. Events that
require ESCRT activity but do not occur at sites targeted by the early ESCRTs must have alternative mechanisms for the recruitment of ESCRT-III and VPS4. One mechanism to directly recruit the late ESCRTs is through the adapter protein ALG-2-interacting protein X (ALIX). ALIX interacts with the ESCRT-I subunit Tsg101 and the ESCRT-III subunit CHMP4 [192, 193]. During ILV formation, ALIX is thought to stabilize the interactions between complexes. However, ALIX also interacts with proteins not associated with the ESCRT machinery. During cytokinesis, centrosomal protein of 55 kDa (Cep55) recruits ALIX to sites of abscission, which results in ESCRT-III recruitment to complete cytokinesis [194]. It is through interactions with adaptor proteins that the activity associated with the late ESCRTs can be redirected for other uses.

**ESCRTs and their role during viral infection**

Many viruses have developed mechanisms to utilize the ESCRT scission machinery during various stages of their replication cycles (Figure 1-6). Examples of viral modulation of the ESCRT machinery will be discussed in this section. To study the role of the ESCRT machinery during virus infection, several approaches have been used to block ESCRT function within the cell and observe how this impacts the replication cycle of the virus. One method is to block ESCRT activity by using RNA interference (RNAi) to knockdown ESCRT subunit expression, which has been demonstrated to reduce the activity of the pathway [157, 195, 196]. Expression of recombinant CHMP proteins fused to large polypeptides such as green fluorescent protein (GFP) will also block the activity of the entire ESCRT pathway [197, 198]. These are considered to be dominant negative forms of the protein since the expression of only one recombinant subunit within the cell is sufficient to disrupt the activity of the endogenous proteins.
Figure 1-6: The ESCRT machinery and virus infection.
Additionally, expression of VPS4 with a catalytically inactive ATPase domain acts in a similar dominant negative fashion as the polypeptide tag on CHMP subunits [199].

Many studies investigating the role of the ESCRT machinery during virus infection have used these tools to study how inhibition of the ESCRTs affects the replication cycle of different viruses. Virtually all reports of viral manipulation of the ESCRTs have been identified using techniques to block the endogenous activity of the ESCRT machinery, but these tools must be used with caution. The ESCRTs, particularly the late-acting ESCRTs, are required for essential cellular functions such as cytokinesis and maintenance of signaling homeostasis through the endosomal degradation pathway [168]. In cell culture models, uncontrolled disruption of the ESCRT activity for prolonged periods of time will result in cell death, and ESCRT knockout animal models are embryonic lethal [200, 201].

Cytotoxic effects associated with a block in ESCRT activity indirectly affect viral replication due to the inability of the cell to support the demands of the viral replication cycle [198]. While RNAi and dominant negative protein expression are powerful molecular tools, inhibition of ESCRT activity must be interpreted with careful consideration of toxic effects on the cell. In the following paragraphs, key themes of viral manipulation of the ESCRT pathway will be discussed. However, the role of ESCRT-dependent extracellular vesicles during viral infection will be described in the next section.

RNA viruses replicate their genome within the cytoplasm, and must avoid host defense mechanisms present within the cell. One way that many RNA viruses achieve this is through the use of membranes from various organelles to generate replication
factories or compartments [202]. These compartments require the deformation of membrane to form vesicular structures that concentrate viral factors and serve as physical barriers to the host defense system. The ESCRT machinery is important for the formation of replication factories during certain viral infections. Brome mosaic virus, an RNA virus in the bromoviridae family, generates replication spherules at the endoplasmic reticulum. Blocking the function of ESCRT-III subunits reduced spherule formation and inhibited subsequent replication of the virus [203]. Similarly, ESCRT-III and VPS4 are incorporated into the replication compartment of tombus virus at peroxisomal membrane, and depletion of ESCRT proteins prevents the formation of this compartment [204, 205]. Interestingly, the ESCRTs are required to form these compartments, but they do not scission the membrane. Membrane scission of the compartments would completely block access to cellular resources used by the virus and prevent the egress of newly formed particles. This suggests a high level of regulation of the ESCRT machinery to facilitate membrane deformation but avoid membrane scission.

The ESCRTs are utilized during various stages of the replication cycle of DNA viruses as well. HSV-1 packages DNA genomes into capsids within the nucleus, and capsids egress through the nuclear envelope to complete assembly in the cytoplasm. Subunits of the ESCRT-III complex are recruited to sites of egress at the nuclear envelope, and depletion of ESCRT subunits causes aberrant nuclear egress [206]. This suggests a role for the ESCRTs in sealing the nuclear envelope following virus budding events. Hepatitis B virus (HBV) also utilizes the ESCRT machinery. Knockdown of ESCRT-II reduced the number of capsids containing packaged genomes [207]. Further analysis revealed that ESCRT-II subunits interact with the HBV capsid protein, and this
interaction could be important for trafficking the core protein to sites of genome packaging.

The ESCRTs are required at many stages of virus infection, but the most extensively studied function of the ESCRTs during viral infection is their role during membrane scission and budding of enveloped viruses. Virus budding and envelopment, like the formation of ILVs, occurs in a mechanism where the virus is budding away from the cytoplasm. Studies with human immunodeficiency virus (HIV) were the first to demonstrate that the ESCRT machinery was required for a virus budding event. It was shown that either siRNA knockdown of the ESCRT-I subunit Tsg101 or the expression of dominant negative VPS4 in HIV-infected cells prevented virus budding and release at the plasma membrane [157]. Since this initial report, there is now an extensive list of viruses known to require the ESCRTs for envelopment [208]

**Viral late domains**

Since viruses acquire their envelope from different membranes within the cell, viruses that utilize the ESCRTs must have mechanisms to recruit this machinery to sites of envelopment. In many instances, this appears to be through viral proteins that directly interact with the ESCRTs and recruit the proteins to budding sites. The first indication that domains in viral proteins were responsible for recruitment of scission proteins to sites of envelopment was during studies of the retrovirus Rous sarcoma virus (RSV). The RSV Gag protein is essential for virus budding, and mutational analysis of the protein found sequences necessary for virus budding at a very late stage in the envelopment process after the viral particle was already associated with the membrane [209]. While the role of the ESCRTs during virus budding had not yet been established, these amino
acid sequences were termed late domains because they were required for such a late step in the budding process [210].

RSV is not the only virus that contains late domain sequences. Mutational analysis of HIV Gag found that a PTAP sequence in the p6 domain of the protein was responsible for the late phenotype [211, 212]. The PTAP region of Gag interacts with the ESCRT-I subunit Tsg101 [157, 195]. This represents a mechanism by which the virus can recruit the ESCRTs to sites of membrane scission through interactions with Tsg101. HIV is not the only virus to utilize this mechanism. Ebola virus also requires the ESCRT machinery for budding, and the viral protein VP40 contains a PTAP late domain sequence that is necessary for the recruitment of Tsg101 to sites of envelopment. Mutation of the sequence blocks the release of virus-like particles [213].

In addition to the PTAP late domain, other mechanisms of ESCRT recruitment have been identified (Figure 1-7). As previously described, the adapter protein ALIX is an important factor that can directly recruit the late ESCRTs [193]. Viral proteins that contain YxxL sequences, where x denotes no specific amino acid requirement, are able to recruit the ESCRT machinery through interactions with ALIX. The M protein of Sendai virus and the NS3 protein of yellow fever virus recruit the ESCRT machinery to sites of envelopment through late domain interactions with ALIX [214, 215].

A third late domain is a PPxY motif that allows interactions with the Nedd4 family of ubiquitin ligases. While Nedd4 family proteins are not directly implicated in the ESCRT pathway, they contribute to ESCRT recruitment through ubiquitination of target proteins at the site of viral envelopment [216]. Further support that ubiquitination serves as a viral mechanism for ESCRT recruitment is hepatitis C virus (HCV) envelopment.
Figure 1-7: Viral late domains recruit the ESCRT machinery to sites of budding and envelopment. Viruses and the ESCRT component that they recruit are shown above. PTAP motifs recruit ESCRT-I through Tsg101. YxxL motifs interact with ALIX and recruit ESCRT-III. PPxY motifs interact with Nedd4 family ligases and recruit the ESCRT machinery through ubiquitination. Hepatitis C virus recruits ESCRT-0 through modulation of the ubiquitination pathway.
HCV requires ubiquitination of the viral protein NS2 to recruit ESCRT-0 to facilitate envelopment [217].

Viruses can also have multiple late domains, and redundancy in the recruitment and utilization of the ESCRT pathway can complicate interpretations about which complexes or domains are truly required during viral infection. An example of this is the budding process of HIV, where various data suggests that Tsg101, ALIX, and Nedd4 can all initiate HIV budding [157, 218, 219]. Since budding and envelopment is such an essential process to viral replication, redundancy likely exists in many viral systems to ensure the envelopment can proceed even in the absence of certain factors.

**Herpesviruses and the ESCRT machinery**

The role of the ESCRTs during herpesvirus envelopment is of particular importance to this study. Studies on the envelopment process of gammaherpesviruses EBV and KSHV are limited, and to this point it is unclear whether the ESCRTs are required. However, the role of the ESCRT proteins during envelopment of the alphaherpesvirus HSV-1 is well defined. Expression of either the dominant negative ESCRT-III subunits or the catalytically inactive VPS4 mutant reduced the amount of infectious virus released from cells [220, 221]. Dominant negative VPS4 expression blocked HSV-1 envelopment at a late budding step, suggesting HSV-1 requires the ESCRTs for budding and envelopment. While HSV-1 requires the late ESCRTs for envelopment, the mechanism of recruitment is still undefined. Inhibition of either Tsg101, ESCRT-II subunits, or ALIX does not prevent viral envelopment [221, 222]. Understanding how HSV-1 recruits the ESCRT machinery may lead to the discovery of new methods of recruitment and new targets for therapeutic intervention.
The ESCRTs are also involved in HCMV infection, but further investigation is needed to understand their role during infection. One study using siRNA to knockdown ESCRT subunits found that knockdown of Tsg101, ALIX, or VPS4 had no effect on HCMV single-step replication [196]. However, a second study found that expression of dominant negative VPS4 in cells slowed virus spread through a cell monolayer [223]. Together, these data suggest that the late-acting ESCRTs may have a role during the replication cycle of HCMV, but their specific function during envelopment was not analyzed.

**ESCRT-independent viral envelopment**

ESCRT proteins are required by most enveloped viruses that have been studied, but it is important to note that not all enveloped viruses require this machinery during their budding process. This appears to be true for influenza virus, which buds using an ESCRT-independent mechanism [224]. The M2 viral protein was sufficient to generate ILVs using an in vitro GUV system. Analysis of infected cells also showed that M2 localized to the bud neck of influenza particles at the late stage of envelopment, suggesting that M2 may facilitate budding in an ESCRT-independent mechanism. Other viruses bud in mechanisms that are independent of the ESCRT machinery even though the alternative mechanism has not yet been identified. This is the case for Semliki Forest virus (SFV), where budding was not inhibited through ubiquitin depletion or expression of dominant negative VPS4 [225]. In summary, enveloped viruses acquire their lipid membrane through distinct mechanisms. Some of these mechanisms include all or a subset of the ESCRT proteins, while other viruses facilitate envelopment in completely ESCRT-independent mechanisms.
Coronavirus envelopment

During the preparation of this dissertation a novel coronavirus, severe acute respiratory syndrome-related coronavirus 2 (SARS-CoV-2) is causing a pandemic with major health and economic impacts [226, 227]. Infection with SARS-CoV-2 can lead to severe respiratory illness with potentially fatal consequences that has been called coronavirus disease 2019 (COVID-19). The outbreak of COVID-19 is ongoing so the consequences of the rapid spread of SARS-CoV-2 cannot be fully appreciated, but the virus has already led to the death of over 100,000 people and overwhelmed healthcare systems across the world [228]. The outbreak has generated an increased need for research related to the biology of coronavirus infection to rapidly bring candidates for antivirals and vaccines to the clinic to control the spread of infection. Given the circumstances, it seems appropriate to provide a brief overview of the details that are currently known about coronavirus envelopment.

Coronaviruses are positive-sense single-stranded RNA viruses that are known to infect mammals and birds [229]. All coronaviruses encode a common set of structural proteins, including: spike (S), membrane (M), and envelope (E) which are found in the viral envelope. Budding and envelopment of viral particles is thought to occur in the endoplasmic reticulum Golgi intermediate compartment prior to egress through the secretory pathway [229]. The E and M proteins are important during viral budding, as it is known that expression of just these two proteins is sufficient to generate the release virus-like particles [230, 231]. However, experimental evidence suggests that the E protein could be involved during late stages of viral assembly and envelopment. Mutations in the E open reading frame are known to affect the membrane morphology of
virions, and prevent the release of viral particles [232, 233]. This and other data led to the model that E is involved during the membrane scission process during viral envelopment [234, 235]. If the E protein is able to facilitate viral budding, it is possible that coronaviruses do not require the ESCRT machinery. However, this has not been directly tested. It will be important to test whether inhibition of ESCRT machinery affects coronavirus envelopment to further understand the requirements during this stage of viral replication.

**Extracellular Vesicles**

**Identification of EVs and their biological relevance**

Cell-to-cell communication is an important regulatory mechanism in response to different environmental situations. Intercellular communication can be achieved in a variety of ways that can have localized or global effects within biological systems. One method of communication is through the release of soluble factors that activate signaling pathways of nearby or distant cells. An example of this is the release of interferons. Infected cells can produce and release interferons that are transferred to nearby cells and activate an antiviral state to control viral replication and spread [236]. This is an effective system that viruses such as HCMV must find ways to subvert to successfully replicate and spread [237]. A second method of cell-to-cell communication is through physical contact between adjacent cells. Cell signaling generated through this method is important to immune signaling through the presentation of viral antigen to immune cells via major histocompatibility complex (MHC) molecules [238, 239]. HCMV encodes a number of
proteins that alter this signaling pathway to help facilitate efficient viral replication and spread [240]. A third mechanism of cell-to-cell communication is through the transfer of signaling molecules in membrane bound vesicles, termed extracellular vesicles (EV). These vesicles can fuse with recipient cells and release their cargo into the cytoplasm of the recipient cell. The importance of this pathway during virus infection is becoming increasingly apparent.

EVs were first reported in the plasma of humans and other animals fifty years ago [241, 242]. These vesicles were identified in other biological fluids and in the medium of cultured cells. Purified EVs were associated with enzymatic activity, suggesting that EVs could have a functional significance in biological systems [243, 244]. Initial studies proposed that vesicles were generated through budding mechanisms that occurred at the plasma membrane of cells. However, electron microscopy analysis identified that EVs are generated from different sites of origin including the release of vesicles through fusion of MVBs at the plasma membrane [245-247]. It was hypothesized that this could be a secondary, or even an unintended way to remove cargo from the cell that was destined for degradation in the lysosome. The importance of EVs as signaling molecules was appreciated when it was identified that EVs could alter the activity of recipient cells that internalized the vesicles. This was first demonstrated by experiments that showed EVs released from cells contained molecules that could stimulate the activity of T cells [248, 249]. This finding led to further investigation of EVs as an important factor in cell-to-cell communication.

While initial studies suggested only the presence of proteins within EVs, it is now known that vesicles can carry a diverse set of cargo (Figure 1-8A).
Figure 1-8: Extracellular vesicle packaging and biogenesis. A) Diagram of the molecular cargo found inside extracellular vesicles. B) Schematic of biogenesis pathways used to generate vesicles with unique cargo from endosomal compartments.
Multiple databases catalog the lipids, proteins, DNA, mRNA, miRNA, and lncRNA that can be found in EVs released from different cell types and conditions [250-252]. As our understanding of EVs has increased, it is now known that they are important for normal homeostatic signaling. During the early stages of development, EVs carry signaling molecules necessary for progenitor cell proliferation, and loss of EV formation in vivo impairs development [253]. Cells undergoing certain types of cell death can release EVs that activate macrophages and may contribute to clearance of cell debris [254]. EVs are also commonly dysregulated in disease states. Alterations in EV signaling can even contribute to disease progression and severity. EVs are also commonly altered in inflammatory states. During exposure to allergens, cells can release vesicles containing allergens that further enhance the activation of mast cells and contribute to an anaphylactic response [255]. Currently, the largest area of EV research is focused on their contribution to cancer. Cancer cells commonly have altered EV cargo that affect signaling when transferred to recipient cells. Cancer cells can release EVs that inhibit immune cell activity, promote angiogenesis, and increase vascular permeability [256, 257]. These changes can prime the tumor niche, or promote tumor progression and metastasis, making this a key area of exploration for diagnostic and therapeutic purposes.

**EV biogenesis**

EV biogenesis pathways are still an area of active investigation. This section will focus on the three well-defined pathways of biogenesis of EVs from the endocytic vesicles based on their relevance to this work (Figure 1-8B). The generation of these vesicles requires the formation of ILVs into an endocytic vesicle that will ultimately fuse with the plasma membrane to release the vesicles as EVs. The cellular machinery best
known for membrane deformation and scission during the formation of ILVs are the ESCRTs. Components of the ESCRT machinery are found inside EVs released from different cell types [258, 259]. A key study found that knockdown of select ESCRT proteins caused a reduction in EVs produced by the cell [260]. The study subsequently found that extracellular levels of certain markers of EVs were also decreased during a knockdown of ESCRT expression, consistent with a block in EV formation. However, extracellular levels of some markers were unaffected or even increased during siRNA knockdown of ESCRT proteins. This suggested that the ESCRT machinery may not represent the only mechanism to generate EVs released from cells. This was further supported by the fact that blocking ESCRT activity had variable effects on EV release depending on the cell type and markers used to measure EVs [261-264].

Lipid composition at the endosomal membrane is also an important regulator of EV formation and release. An initial study using a neuronal cell line found that EV release of proteolipid protein occurred in an ESCRT-independent manner [265]. The purified EVs were enriched for the lipid ceramide. Inhibition of neutral sphingomyelinase (nSMase), the enzyme required for the formation of ceramide at endosomal membranes, prevented the release of EVs containing the proteolipid protein marker. The formation of ceramide is a common vesicle biogenesis mechanism, as inhibition of other classes of sphingomyelinases also prevented the budding of vesicles from the plasma membrane [266]. These findings have allowed sphingomyelinase inhibitors to be widely used as EV inhibitors to study the function of EVs in various experimental systems [265, 267, 268].

A third method of EV biogenesis is through the regulation of certain membrane proteins found in EVs. Regulators of EV biogenesis were identified in a screen of cell
lines that correlated intracellular mRNA expression of genes with the number of EVs released by the cell [269]. In this screen, mRNA levels of the gene encoding CD63 were positively correlated with increased vesicle production. This was confirmed when CRISPR knockout of CD63 caused a reduction in EV release. CD63 is a tetraspanin commonly enriched on the surface of EVs, and its role in the regulation of EV formation and release has been clearly demonstrated in other studies [270, 271]. While CD63 is important for EV release in certain contexts, the mechanism is still unclear. However, some indications suggest that CD63 may be an important regulator of autophagic flux, which may have effects on the number of EVs released [272].

**EV composition and cargo selectivity**

The effect an EV has on a recipient cell is dependent on the cargo packaged inside the vesicle. Since vesicles are generated in the cytoplasm, an early theory was that EVs likely contained factors in similar abundance to what could be found in the cytoplasm of the cell of origin. However, analysis of components released into EVs has revealed that this is a much more selective process. While mechanisms involved in the selective packaging of EV cargo are still being discovered, a few methods of targeting protein and miRNAs to sites of EV assembly are described below.

One mechanism for selective packaging is through the interaction with proteins associated with EV biogenesis. Late domains were described as a viral mechanism for recruitment of the ESCRT machinery to sites of envelopment. This also appears to be a pathway for selective recruitment into EVs. Cellular proteins that contain late domains were found to be selectively incorporated into EVs, and expressing a recombinant GFP molecule that included the Tsg101-interacting PTAP motif was sufficient to target the
recombinant GFP for EV packaging [273]. Interactions with proteins associated with EV biogenesis likely cause these molecules to be in close proximity to the newly forming vesicle and result in incorporation into EVs. This is also true for proteins that interact with CD63 [274, 275]. This indicates that different biogenesis pathways may generate vesicles that contain unique cargo based on their mechanism of packaging. In fact, some evidence suggests that EVs generated through ESCRT, CD63, or ceramide-dependent pathways carry a unique set of cargo [260, 265]. Posttranslational modification of proteins also appears to target proteins for EV packaging. While the mechanism is not completely understood, proteins containing a ubiquitin-like 3 (UBL3) protein modification are highly enriched in EVs, and preventing this modification inhibits their incorporation [276].

Packaging of miRNAs into EVs is also thought to be a selective process. It is well established that intracellular miRNA abundance does not correlate with miRNA abundance detected in EVs [277, 278]. The process of selective incorporation appears to be highly dependent on miRNA interactions with certain RNA binding proteins found in EVs. Heterogeneous nuclear ribonuclear protein A2B1 (hnRNPA2B1), synaptotagmin binding cytoplasmic RNA interaction protein (SYNCRIP also known as hnRNPQ), and Y box binding protein 1 (YBx1) are RNA binding proteins that selectively package miRNAs into EVs [279-281]. While all three proteins have defined roles in the nucleus, knockdown of these proteins resulted in altered miRNA profiles found in EVs. Closer examination identified that a small portion of these RNA binding proteins are in the cytoplasm and are incorporated into EVs. The RNA binding proteins interact with select miRNAs through specific binding sequences that facilitate packaging of the miRNAs into
EVs. The mechanism mediating incorporation of the RNA binding protein into EVs, and where the RNA binding protein and miRNA interact are still unknown. Regardless, this data provides a pathway for miRNA incorporation through the interaction with certain RNA binding proteins.

**EVs and virus infection**

Virus infection can alter the activity of the EV pathway through various mechanisms. The changes caused by virus infection cause diverse effects in the recipient cell that can inhibit or enhance virus replication and spread. Many examples of viral manipulation of the EV pathway exist, but the effects on the EV pathway ultimately result in altered EV composition. A major discovery in the field of virology was that certain RNA viruses could package their genome into EVs that were released from infected cells. The release of “infectious EVs” that no longer contain the same surface antigens and entry receptors as the viral particle has important implications for immune evasion and cell tropism. Infectious genomes that are now enclosed in membrane that only contain host proteins are no longer recognizable by the adaptive immune response. In the case of hepatitis A virus (HAV) and HCV, infected cells release “infectious EVs” that are resistant to neutralizing antibody [282, 283].

The mechanisms of EV entry into recipient cells is still very much unknown. Entry into recipient cells can range from fusion at the plasma membrane to receptor-mediated endocytosis. Importantly, these mechanisms can be different than what is used by viral entry receptors. Enclosing the viral genome in an EV can alter entry mechanisms and cell tropism. Enterovirus-71 (EV71) and JC polyomavirus (JCPyV) infected cells release “infectious EVs” that are now capable of infecting cells that the
viral particle cannot [284, 285]. It is becoming increasingly apparent that “infectious EVs” have important clinical implications.

Aside from incorporation of the infectious genome into EVs, virus infection can alter the composition of the cargo released from infected cells in other ways that have important implications for the immune response and pathogenesis. EVs released from simian immunodeficiency virus and RSV-infected cells contain altered miRNA profiles that increase the inflammatory response when transferred to recipient cells [286, 287]. This may contribute to increased pathogenicity of infection. Altered composition during infection may also be a host defense mechanism during certain viral infections. EVs released during HCV infection stimulate interferon stimulated gene expression in dendritic cells and represent an important mechanism for controlling virus spread [267].

Members of the herpesvirus family are also known to modulate the EV pathway during infection. EVs released during HSV-1 infection contain viral and cellular miRNAs [270, 288]. Interestingly, these EVs also contain innate immune sensing proteins that are transferred to recipient cells and slow virus spread [270]. Altering the levels of CD63 within infected cells affects this phenotype, suggesting that the inhibitory EVs are generated in a CD63-dependent manner [270, 271]. KSHV and EBV also alter the composition of cargo packaged into EVs. Infected cells contain viral proteins and viral miRNAs that alter key cell signaling pathways involving cell migration, adhesion, and metabolism [289-291]. These changes are thought to prime the tumor microenvironment during oncogenesis associated with both viruses. Interestingly, EVs released from B cells infected with EBV may also be important for enhancing transmission to epithelial cells [292]. HCMV is also known to package viral proteins and miRNAs into EVs released
from infected cells [293-295]. While one study demonstrated that EVs were capable of transferring viral antigen to T cells, the full contribution of EVs to HCMV infection is not yet determined. Thus, EVs carry important signaling molecules between cells, and both viruses and the infected cells utilize EVs as a way to either promote or control infection.

**Hypothesis**

The ESCRTs are utilized during the replication cycle of many viruses. HCMV also utilizes this machinery, but how the ESCRTs enhance virus replication and spread is unknown. The focus of this work was to determine the role of the ESCRTs during HCMV infection. Chapter 2 tests the hypothesis that the ESCRTs are required for HCMV envelopment. The results in chapter 2 led to the new hypothesis that EVs are important for HCMV spread, which is tested in chapter 3. Together, this work describes how HCMV modulates EV biogenesis machinery to release EVs from infected cells that enhance virus spread.
Chapter 2: Nonenvelopment Role for the ESCRT-III Complex during HCMV Infection

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Abstract

Secondary envelopment of HCMV occurs through a mechanism that is poorly understood. Many enveloped viruses utilize the ESCRTs for viral budding and envelopment. Although there are conflicting reports on the role of the ESCRT AAA-ATPase protein VPS4 in an HCMV infection, VPS4 may act in an envelopment role similar to its function during other viral infections. Because VPS4 is normally recruited by the ESCRT-III complex, we hypothesized that ESCRT-III subunits would also be required for HCMV infection. We investigated the role of ESCRT-III, the core ESCRT scission complex, during the late stages of infection. We show that inducible expression of dominant-negative ESCRT-III subunits during infection blocks endogenous ESCRT function, but does not inhibit virus production. We also show that HCMV forms enveloped intracellular and extracellular virions in the presence of dominant-negative ESCRT-III subunits, suggesting that ESCRT-III is not involved in the envelopment of HCMV. We also found that similar to ESCRT-III, inducible expression of a dominant-negative form of VPS4A did not inhibit the envelopment of virions or reduce virus titers. Thus, HCMV does not require the ESCRTs for secondary envelopment. However, we found that ESCRT-III subunits are required for efficient virus spread. This suggests a role for ESCRT-III during the spread of HCMV that is independent of viral envelopment.
**Importance**

HCMV is a prevalent opportunistic pathogen in the human population. HCMV infection in neonatal and immunocompromised patients can cause severe and possibly life-threatening complications in these at-risk patients. It is important to define mechanisms of the viral replication cycle to identify potential targets for new therapies. Secondary envelopment, or acquisition of the membrane envelope, of HCMV is a mechanism that needs further study. Using an inducible fibroblast system to carefully control for the toxicity associated with blocking ESCRT-III function, this study determines that the ESCRT proteins are not required for viral envelopment. However, the study does discover a non-envelopment role for the ESCRT-III complex in the efficient spread of the virus. Thus, this study advances our understanding of an important process essential for the replication of HCMV.
Introduction

HCMV is a herpesvirus of clinical significance for immunosuppressed individuals and developing fetuses. One essential replication process, secondary envelopment or acquisition of the membrane envelope, remains largely uncharacterized with very little known about the molecular details of this process. Viruses have adapted a variety of strategies for obtaining their envelope layer. In some cases, for example influenza A and the M2 protein, the process is driven entirely by viral products [224]. In other cases, envelopment requires the coordinated effort of both viral and cellular proteins. Due to the nature of the ESCRT machinery to promote budding outward from the cytoplasm, these cellular complexes are often recruited by viral proteins as part of the envelopment process, as was first reported for HIV-1 [157]. The ESCRTs were originally described in the formation ILVs of MVBs, in which the ESCRT complexes are sequentially recruited to promote cargo clustering and vesicle formation. These complexes, recruited in sequential order, include ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III and the VPS4 AAA-ATPase. In the context of viral budding, different subsets of these ESCRT complexes may be recruited. This appeared to be the case for HCMV, in which two independent studies reported that two ESCRT proteins, ALIX and Tsg101, are not required for production of infectious virions [196, 223]. While these studies agreed that the aforementioned ESCRT complexes are not required, they differed with regard to the requirement of the downstream ESCRTs for productive HCMV infection (summarized in Figure 2-1A).
Figure 2-1: High levels of dominant negative ESCRT-III subunits prevent initiation of HCMV infection. A) Model of ESCRT recruitment during ILV formation. B) Virus titers of parental control, GFP, or CHMP6-GFP expressing viruses at 96 hpi (MOI of 3) in the presence or absence of doxycycline (Dox). C) Immunofluorescence staining of immediate early proteins (red) 9 dpi (MOI of 0.05) in the presence of Dox. D) Immunofluorescence staining of pp28 (red) at 96hpi in cells infected with a virus expressing CHMP6-GFP or a control virus at 96hpi. Nuclei were labeled with DAPI (blue). Scale bar: 10μm. E) Western blot analysis of viral proteins pUL44, pp150, and pp28 at 96 hpi (MOI of 3) from fibroblasts infected with GFP or CHMP6-GFP expressing viruses. F) Virus titers of CHMP4C-GFP and CHMP2A-GFP expressing viruses. All virus titer data is from three independent experiments.
In one case, siRNA knockdown of the late ESCRT AAA-ATPase VPS4 does not affect HCMV titers [196], while the other reported that dominant-negative constructs of both VPS4A and its associated factor CHMP1A block infection [223]. Because CHMP1A, along with its binding partner IST1, modulates a subset of VPS4 activities [296], a role for both VPS4A and CHMP1A during HCMV infection, specifically at the envelopment step, seemed like a probable model. Although a specific role for VPS4A and CHMP1A in envelopment was not investigated, from this study it was proposed that, similar to other viruses, HCMV utilizes a subset of the ESCRT machinery to promote envelopment.

Consistent with a role in envelopment, both VPS4A and CHMP1A are recruited to the cVAC during infection [90]. This recruitment may be mediated directly by a viral protein, or by the ESCRT-III complex akin to VPS4 recruitment during ILV formation. ESCRT-III consists of four core subunits (CHMP6, CHMP4, CHMP3, CHMP2) that oligomerize on membrane to form a scission-promoting complex (Figure 2-1A). CHMP6, the first subunit recruited during ILV formation, binds to ESCRT-II and initiates recruitment of the other ESCRT-III subunits and oligomer formation. HCMV may utilize ESCRT-III to recruit VPS4 during infection. The viral protein UL71 and its interacting protein UL103 have recently become well established for their role in envelopment [87, 105, 106, 108]. Furthermore, alphaherpesvirus homologues UL7 and UL51 also play a role in virus assembly and spread [297-299]. Thus, UL71 may function by recruiting the ESCRT-III complex, which subsequently recruits VPS4. Alternatively, UL71 may directly recruit VPS4. As the core ESCRT-III subunits had not yet been investigated during HCMV infection, we began our study with the presumption that they are involved in envelopment. Surprisingly, not only did we discover that ESCRT-III was not required...
for production of infectious virions, we also found that VPS4 is not involved in production of infectious virions. This is consistent with the original report in which VPS4 actually had a slight inhibitory effect on infectious titers [196]. While our findings provide definitive evidence that HCMV does not require the ESCRT machinery for envelopment, we did find that blocking the ESCRT pathway decreased the efficiency of virus spread. This indicates an alternative function for the ESCRT pathway in the mechanism of HCMV spreading, outside of a direct role in virion production.

Results

To investigate whether HCMV required the ESCRT-III complex for infection, we began by testing a role for CHMP6, the first ESCRT-III subunit recruited in ILV formation (Figure 2-1A). Taking advantage of the dominant negative effect that ESCRT-III subunits tagged with fluorophores have on complex activity [198], we engineered the HCMV genome to express CHMP6-GFP to measure its effect on virion production. This approach ensures that every infected cell expresses CHMP6-GFP, and to regulate its expression, we placed it under control of an inducible promoter. As a control, we generated a virus expressing only GFP under control of the same inducible promoter. After induction of CHMP6-GFP, we observed a 2-log reduction in infectious virions (cell-associated plus extracellular) recovered at 96 hpi when compared to the GFP or AD169 parental control viruses (Figure 2-1B). Accordingly, virus spread was also significantly reduced and in some cases was limited to single, isolated cells (Figure 2-1C). In contrast, the control virus spread throughout nearly the entire monolayer.
We next sought to correlate this block in infection to envelopment by first determining whether viral proteins were being properly trafficked to the cVAC despite the block to the ESCRT pathway. Surprisingly, we could detect very little of the late viral protein and cVAC marker, pp28, by immunofluorescence analysis of cells that were infected for 96 hours in the presence of the TET-inducer, doxycycline (Figure 2-1D), which we confirmed by western blot analysis (Figure 2-1E). Additionally, analysis of other viral proteins revealed that almost no pp150 and a markedly reduced amount of pUL44 was present (Figure 2-1E), indicating the inability to initiate a robust infection in the presence of the dominant negative CHMP6 protein. Expressing ESCRT-III dominant negative constructs at high levels results in cell toxicity and similar to our observation with HCMV proteins, reduces expression of the retroviral Gag protein [198]. The significant reduction in viral protein production suggested that expressing the dominant negative ESCRT-III subunits from the viral genome resulted in a level of expression that perturbed cellular conditions sufficiently to prevent a robust infection from establishing. Even in the absence of doxycycline induction, we observed a decrease in both infectious virus titers and levels of the viral protein pp28 (Figures 2-1B&E). We believe this is due to leaky expression from the high number of copies present after HCMV genome replication. Accordingly, CHMP6-GFP protein can be detected by western blot analysis in both the presence and absence of doxycycline (Figure 2-2B). While we cannot rule out that this phenotype was due to a second site mutation in the CHMP6-GFP expressing virus, we do not favor this possibility as similar results were observed using viruses engineered to express other ESCRT-III subunits, CHMP4C-GFP and CHMP2A-GFP and titers were further reduced upon dox-induction (Figure 2-1F). These results emphasized
the importance of tightly regulating dominant negative expression and revealed the need for an alternative approach to assess the role of ESCRT-III subunits during infection.

To better control expression of the ESCRT-III dominant negative subunits, we utilized a lentivirus system with expression of the subunits under control of an inducible promoter. Following lentivirus transduction of both the inducible dominant-negative ESCRT-III construct and a Tet activator, cells were passaged three times under selection to generate a population of cells containing both the Tet activator protein and the dominant-negative expression vector, ensuring that the entire population of infected cells would express both desired constructs. Using this system, we generated cells expressing GFP and dominant negative versions of five of the seven mammalian ESCRT-III subunits (CHMP6, CHMP4B&C, CHMP3 and CHMP2A), and confirmed the inducibility of construct expression in the presence of doxycycline by monitoring the presence of GFP (Figure 2-2A). We observed expression of GFP (or the corresponding CHMP-GFP) in greater than 90% of induced cells. Importantly, we could detect no GFP fluorescence in the absence of doxycycline, (Figures 2-2A & 2B). However, the expression level of CHMP6-GFP after induction was reduced when compared to levels expressed from the genome, with or without doxycycline. While this may be beneficial for avoiding the adverse effects on infection that we observed with the elevated dominant negative levels, we were concerned that we may not be expressing our constructs at a level sufficient to block ESCRT function. Western blot analysis confirmed that the dominant-negative GFP-tagged constructs expressed in the transduced fibroblasts were in fact in excess over the endogenous protein (Figure 2-2C).
Figure 2-2 ESCRT function is inhibited in inducible ESCRT-III fibroblasts. A) GFP expression of inducible fibroblasts expressing GFP, CHMP6-GFP, CHMP4B-GFP or CHMP4C-GFP 24h post dox addition. Nuclei were labeled with DAPI (blue). Scale bar: 10 μm. B) Western blot analysis of HCMV infected cells expressing CHMP6-GFP from the HCMV genome (HCMV) or in fibroblasts following lentiviral transduction (Lenti). Dox was present throughout the entire infection (96 hrs). C) Western blot analysis of endogenous and GFP-tagged CHMP6, CHMP4B and CHMP4C at 96 hpi after dox induction for 0, 48, 72, or 96 hours. NS, non-specific band detected by CHMP4B antibody; *, unknown band detected by CHMP6 only in dox-induced samples. D) EGF degradation in ESCRT-III dominant-negative (green) selected fibroblasts. EGF (red) was imaged 3 hours after 30 min pulse in the presence or absence of Dox. Nuclei are labeled with DAPI. Scale bar: 10 μm. E) HSV-1 infection of fibroblasts expressing GFP, CHMP6-GFP, or CHMP4-GFP at 24hpi (MOI of 5). Dox was added 24h prior to infection. Significant differences (p<.05) between samples are marked with asterisk. Infections are from three independent experiments.
Furthermore, to ensure that our system was capable of expressing dominant negative ESCRT-III subunits at a level sufficient to block pathway function, we used an assay that monitors the degradation of a fluorescently labeled EGF reporter. Upon binding its receptor, EGF is endocytosed and delivered to the lysosome for degradation in an ESCRT-dependent manner. In the absence of doxycycline, EGF was degraded in all samples. However, after doxycycline induction clear EGF punctate were present in all of the ESCRT-III dominant negative samples, but not the GFP sample, indicating a block in the ability to degrade EGF (Figure 2-2D). This suggests that ESCRT function was successfully inhibited.

While this result indicates a successful block to ESCRT function, we sought a second assay to confirm that our cells expressing the dominant negative constructs were functioning as expected. ESCRT-III subunits have previously been found to be important for HSV-1 replication [220, 221]. Using our induction system, we next showed that HSV-1 titers were reduced when the expression of CHMP6-GFP, CHMP4B-GFP or CHMP4C-GFP was induced in the respective dominant negative selected fibroblasts (Figure 2-2E). The block was more potent with the CHMP4 isoforms than with CHMP6-GFP, a similar observation to what was originally published [221]. Importantly, there was no difference in titers after induction of the GFP control. These results validate that ESCRT-III function is in fact inhibited in our system.

The uncontrolled expression of the dominant negative proteins from the viral genome resulted in protein levels sufficient to cause cytotoxicity. Viral protein expression was significantly reduced, as the infection was unable to proceed under these cytotoxic conditions (Figure 2-1). To ensure that our lentivirus system sufficiently controlled
expression of the ESCRT-III dominant negatives to avoid generating conditions too toxic for an infection to progress, we examined the expression and localization of viral proteins. We harvested lysates from our HCMV-infected dominant negative containing fibroblasts at 96 hpi and found no difference in the level of the viral proteins pUL44, pp150, or pp28 regardless of the duration of induction (Figure 2-3A). Furthermore, there was no difference in pp28 or gB localization after induction of either CHMP6-GFP or CHMP4B-GFP (Figure 2-3B), suggesting that viral proteins were able to traffic to what appears to be a properly formed cVAC in the absence of a functional ESCRT-III pathway. This important observation confirms that blocking the ESCRT pathway does not alter the trafficking of viral factors; however, it does not reveal whether a block is occurring for cytoplasmic envelopment, a process that occurs temporally after the proper formation of and trafficking of viral factors to the cVAC.

One final test of our system was to monitor the status of the cells when the ESCRT-III pathway was inhibited. Using three different cell viability assays based on three different parameters (membrane permeability, NADPH and ATP levels), we found that blocking ESCRT-III often resulted in a slight but significant drop in cell viability after induction of the dominant negative subunits. This observation illustrates the importance of tightly controlling dominant negative ESCRT-III expression to a level and duration sufficient to block pathway function without inducing cell toxicity (Figure 2-3C-F).
Figure 2-3: HCMV viral protein expression and cVAC formation are unaffected by dominant negative ESCRT-III subunits. A) Western blot analysis of viral proteins 96 hpi (MOI of 3) in fibroblasts expressing CHMP6-GFP, CHMP4B-GFP or CHMP4C-GFP. Hours indicate duration of dox induction. B) Immunofluorescence staining for pp28 (red) and gB (green) 96hpi. Fibroblasts were treated with dox for 48h prior to fixation. Nuclei were labeled with DAPI. Scale bar: 10 μm. C-E) Cell viability assays on fibroblasts expressing GFP, CHMP6-GFP, CHMP4B-GFP or CHMP4C-GFP 96 hpi (MOI of 3) as measured by C) Trypan Blue D) XTT and E) CellTiter-Glo. For each cell type, viability is shown relative to no dox treatment. Significant differences (p< 0.05) between samples are marked with asterisk. All viability assays are from at least three independent experiments.
Having established a system in which ESCRT-III function was inhibited without high levels of cell toxicity, we next investigated whether ESCRT-III was required for productive HCMV infection. Expression of the dominant negative constructs was induced for either 96 (doxycycline added at the time of infection), 72, or 48 hours. Regardless of the time of induction, the presence of the CHMP6 dominant negative protein had no significant effect on total (cell-associated plus extracellular) infectious virus titers (Figure 2-4A) and therefore does not appear to play a role in the HCMV replication cycle. During ILV formation, a major role of CHMP6 is to bind to ESCRT-II to initiate ESCRT-III oligomerization. While a role for ESCRT-II has not been investigated during HCMV infection, it is known that ESCRT-I is not required. Thus, it is possible that HCMV bypasses the need for both the upstream ESCRT complexes and CHMP6 by directly recruiting CHMP4 to initiate the ESCRT-III polymer, which in turn recruits VPS4. In a similar manner, HIV does not require the ESCRT-II complex or CHMP6 for budding as CHMP4 is recruited in an alternative manner [155].

To investigate a role for CHMP4, we used dominant negative versions of two of the CHMP4 isoforms, CHMP4B and CHMP4C. Surprisingly, neither isoform affected viral titers (Figure 2-4B). Thus, neither CHMP6 nor CHMP4 is required for producing infectious HCMV virions, in contrast to HSV-1 (Figure 2-2D). Similarly, virus titers were not affected in the presence of CHMP3 or CHMP2A dominant negatives (Figure 2-4C), although these two isoforms were not subjected to all of the same extensive tests measuring toxicity and the functional block as the CHMP6 and the CHMP4 isoforms. However, western blot analysis confirms that the dominant negative CHMP2A protein was in abundance over endogenous levels (Figure 2-4D).
Figure 2-4: ESCRT-III is not required for productive HCMV infection. Virus titers 96hpi (MOI of 3) of fibroblasts expressing A) GFP, CHMP6-GFP, B) CHMP4B-GFP, CHMP4C-GFP, and CHMP2A-GFP with HCMV strain AD169. D) Western blot analysis of endogenous and GFP-tagged CHMP3 and CHMP2A at 96 hpi after dox induction for 0, 48, 72 or 96 hours. Additional inset shows mock and 96 hpi lysates probed with CHMP2A antibody. NS, non-specific band detected by CHMP3 antibody. E) Intracellular and extracellular virus titers (96 hpi) on fibroblasts expressing GFP, CHMP6-GFP, or CHMP4B-GFP, infected with HCMV AD169 in the presence or absence of dox (48hr induction). F) Virus titers 96hpi on fibroblasts with HCMV strain TB40/E. p-values indicate no significant differences between samples (p>0.05). Titers are from at least three independent experiments. G) Micrographs depicting localization of CHMP-GFP proteins in relation to pp28 (red). Scale bar: 10 μm.
In contrast, endogenous levels of CHMP3 were similar to CHMP3-GFP, indicating the possibility that CHMP3-GFP levels may not be sufficient to block complex function. Nonetheless, we could detect no reduction in virus titers under conditions where ESCRT-III function was shown to be inhibited.

The above titer data was a combination of both cell-associated and extracellular virus. To show that there was no reduction in the release of extracellular virus, we next harvested cell-associated and extracellular virus separately. We found no difference in the release of infectious virions from uninduced cells and cells expressing GFP, CHMP6-GFP, or CHMP4B-GFP (Figure 2-4E). Taken together, these observations are not consistent with the hypothesis that ESCRT-III is involved in envelopment. To show that these results were not specific for the laboratory strain AD169, we infected our GFP, CHMP6, and CHMP4B-selected fibroblasts with HCMV strain TB40/E. We found that induction of the dominant negative CHMP6 or CHMP4B subunits after infection with TB40/E did not affect production of infectious virions (Figure 2-4F), confirming our observation that HCMV does not require ESCRT-III for a productive infection. In support of this observation, we found that of the ESCRT-III subunits investigated, only CHMP6 was recruited to the assembly compartment (Figure 2-4G). Thus, HCMV does not recruit ESCRT-III to mediate the envelopment step.

While these observations suggest that ESCRT-III is not involved in envelopment, we next used electron microscopy (EM) to examine cytoplasmic and extracellular virions in the presence of our dominant-negative ESCRT-III subunits. Expression of CHMP6 or CHMP4B dominant negative proteins did not affect envelopment, as properly enveloped virions in the cytoplasm and extracellular space were prevalent (Figure 2-5A).
Figure 2-5: Dominant negative ESCRT-III does not inhibit HCMV secondary envelopment. A) Electron micrographs of enveloped intracellular and extracellular virions in fibroblasts expressing GFP, CHMP6-GFP, or CHMP4B-GFP at 96 hpi with AD169 (MOI of 3). Fibroblasts were treated with dox for 48h prior to fixation. B) Graph showing percentage of membrane-associated capsids that have not completed envelopment compared to percentage of fully enveloped capsids. N=182 (GFP), 190 (CHMP6) and 181 (CHMP4B).
Quantitative analysis of the EM images revealed no difference among the samples in the percentage of membrane-associated capsids undergoing envelopment and fully enveloped capsids (Figure 2-5B). This is consistent with the absence of any significant reduction in virus titers. Since blocking a single subunit has the net effect of blocking the entire ESCRT-III pathway, our data strongly supports that ESCRT-III is not required for production of infectious virions and does not mediate envelopment. However, we cannot completely rule out a contribution from isoforms that were untested (CHMP4A, CHMP2B) or undertested (CHMP3, CHMP2A).

**VPS4 is not required for production of infectious HCMV virions.**

Our results indicate that ESCRT-III is not required for HCMV infection. However, VPS4, which is normally recruited by ESCRT-III, has been shown to localize to the viral cytoplasmic assembly compartment [90] and may play a role in HCMV infection, although conflicting reports about a role for VPS4 exist [196, 223]. Since there are conflicting reports about the role of VPS4 during an HCMV infection, we first wanted to confirm the VPS4 phenotype utilizing our inducible system in which we can block function without inducing toxicity to cells. Accordingly, we generated cells expressing GFP-fusions of wildtype VPS4A or VPS4A_{E228Q}, which blocks ATP hydrolysis, acts in a dominant negative manner, and inhibits budding of HIV-1 [157]. Expression of both constructs was induced in the presence of doxycycline (Figure 2-6A). We next showed that we were able to successfully block the ESCRT pathway, as shown by the accumulation of a plasma membrane protein, EphA2, which is endocytosed and targeted for degradation during HCMV infection [300].
Figure 2-6: VPS4A is not required for HCMV envelopment. A) Fibroblasts transduced with inducible GFP-VPS4A WT or VPS4A\textsubscript{E228Q} 24h post dox treatment. Nuclei were labeled with DAPI. Scale bar: 10 μm. B) Western blot analysis of cellular protein EphA2, viral proteins pUL44, pp150, and pp28 at 96hpi with AD169 (MOI: 3) in fibroblasts expressing WT VPS4A (WT) and VPS4A\textsubscript{E228Q} (DN). (C) Virus titers on fibroblasts expressing WT and DN VPS4A 24 hpi with HSV-1. Dox was added to cells 24h prior to infection. * p<0.05. Virus titers of (D) HCMV AD169 and (E) TB40/E at 96 hpi (MOI: 3) from fibroblasts expressing WT or DN VPS4A. p-values indicate no significant differences between samples (p>0.05). Viral titers are from three independent experiments. (F) Electron micrographs of enveloped intracellular and extracellular virions from VPS4A\textsubscript{E228Q} cells infected with HCMV AD169 96 hpi (MOI: 3). Dox was added to cells for 48h prior to fixation.
EphA2 levels were significantly increased after induction of the dominant negative, but not wildtype, form of VPS4A (Figure 2-6B). As a confirmation of the block in VPS4 function, infection of the VPS4AE228Q fibroblasts with HSV-1 resulted in a 1-log reduction in the production of infectious HSV-1 virions (Figure 2-6C), similar to what has been reported [221]. We next measured cell-associated and extracellular infectious HCMV virions and, contrary to our hypothesis, found that blocking VPS4 function did not reduce HCMV titers after infection with either the AD169 or TB40/E strains (Figure 2-6D&E). This was particularly surprising because after induction of the VPS4A dominant negative construct for any amount of time, we noticed a reduction in steady state levels of pUL44, pp150, pp28, and gB (Figure 2-6B). This suggests that prolonged exposure to the VPS4A dominant negative construct was beginning to adversely affect cell fitness, which was confirmed with an observed decrease in cell viability in two of our three assays (data not shown). Importantly, despite the reduction in viral steady-state protein levels and cell viability, we observed no decrease in infectious virus titers. If anything, there was a slight increase in titers with the AD169 strain, as was originally reported using siRNA against VPS4 [196].

ESCRT-III has a non-envelopment role in the spread of HCMV

While our results are consistent with VPS4 not being required for the production of infectious virions, and hence capsid envelopment, we were interested in the conflicting reports for a role for VPS4 during infection [196, 223]. One explanation was that the transfection of the dominant negative constructs led to cell toxicity and the inability to support an infection. This is consistent with our results showing that cells are sensitive to both the level and duration of dominant negative expression. A second possibility could
be the differences in the MOI. In our study and the siRNA knockdown study reporting no
effect for VPS4 [196], high MOIs were used. In contrast, a phenotype for VPS4 was
found using a low MOI monitored by antigen spread [223]. Therefore, we used a low
MOI and asked whether blocking ESCRT-III would affect the spread of HCMV, similar
to what was reported for VPS4. To avoid toxicity concerns with expressing our dominant
negative ESCRT-III subunit in the entire population for an extended period of time, we
modified our system slightly and placed the Tet activator protein in the HCMV genome.
Thus, although the entire population of cells contained the dominant negative ESCRT-III
construct and was exposed to doxycycline, only the infected cells would contain the Tet
activator and be able to induce expression of the dominant negative construct. After a low
MOI infection, we fixed cells at 3, 6, 9, 12, and 15 days post infection and stained for
expression of the major immediate early proteins. We then calculated the percent of the
monolayer that was infected. In cells expressing only GFP, the infection spread
throughout the monolayer in both the presence and absence of doxycycline
(Figure 2-7A&B). In contrast, in the presence of CHMP6-GFP or CHMP4B-GFP, there
was a delay in spread at both 9 and 12 days post infection. For both constructs, however,
this delay was overcome and infection was indistinguishable from the uninduced cells at
15 days post infection. A similar result was observed for both CHMP4C and CHMP3
(Figure 2-7C). Thus, ESCRT-III improves the efficiency of spread but does not appear to
be essential for HCMV replication.
Figure 2-7: Dominant negative ESCRT-III subunits slow HCMV spread during low MOI infection. Low MOI (0.05) infection of inducible GFP, CHMP6-GFP, and CHMP4B-GFP fibroblasts with AD169 Tet activator HCMV. A) Immunofluorescence staining for immediate early protein (red) 3, 9, and 15 dpi. Dox was added to cells at the beginning of infection with fresh dox added every 3 days thereafter. B) Percentage of monolayer infected was calculated by comparing DAPI and immediate early staining fluorescence within the monolayer from three independent experiments. C) Percentage of monolayer infected in fibroblasts expressing CHMP4C-GFP and CHMP3-GFP during low MOI infection treated with dox as described for A).
Chapter 3: Human Cytomegalovirus Utilizes Extracellular Vesicles to Enhance Virus Spread

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Abstract

HCMV manipulates cellular processes associated with secretory pathways within an infected cell to facilitate efficient viral replication. However, little is known about how HCMV infection alters the surrounding cellular environment to promote virus spread to uninfected cells. EVs are key signaling molecules that are commonly altered in numerous disease states. Previous reports have shown that viruses commonly alter EVs, which can significantly impact infection. This study finds that HCMV modulates EV biogenesis machinery through upregulation of the ESCRT proteins. This regulation appears to increase the activity of EV biogenesis, since HCMV-infected fibroblasts have increased vesicle release and altered vesicle size compared to EVs from uninfected cells. EVs generated through ESCRT-independent pathways are also beneficial to virus spread in fibroblasts, as treatment with the EV inhibitor GW4869 slowed the efficiency of HCMV spread. Importantly, the transfer of EVs purified from HCMV-infected cells enhanced virus spread. This suggests that HCMV modulates the EV pathway to transfer proviral signals to uninfected cells that prime the cellular environment for incoming infection and enhance the efficiency of virus spread.
Importance

HCMV is a herpesvirus that leads to serious health consequences in neonatal or immunocompromised patients. Clinical management of infection in these at-risk groups remains a serious concern even with approved antiviral therapies available. It is necessary to increase our understanding of the cellular changes that occur during infection and their importance to virus spread. This may help to identify new targets during infection that will lead to the development of novel treatment strategies. EVs represent an important method of intercellular communication in the human host. This study finds that HCMV manipulates this pathway to increase the efficiency of virus spread to uninfected cells. This finding defines a new layer of host manipulation induced by HCMV infection that leads to enhanced virus spread.
Introduction

HCMV is a leading cause of congenital viral infection, which can result in numerous complications for the developing fetus and newborn. Infection of immunocompromised individuals is also a significant source of disease, particularly in solid organ and hematopoietic cell transplant recipients, where virus infection is commonly detected in patients [15-17]. There is a need to understand the molecular mechanisms of infection to provide new therapeutic targets to combat antiviral resistance and toxicity associated with the currently approved therapies.

HCMV expresses over 200 proteins and noncoding RNAs from a large DNA genome [5, 6]. This allows extensive modulation of the host to facilitate efficient replication and spread of the virus. The temporal expression of viral factors during infection supports the various stages of a prolonged replication cycle. Most studies have focused on how this regulation impacts viral replication within the infected cell. However, whether HCMV alters intercellular communication between infected and surrounding uninfected cells is less understood. HCMV causes a dramatic reorganization of cellular membranes and organelles associated with the secretory system during the formation of the cVAC, which results in altered vesicle trafficking [89, 90, 301]. Viral miRNAs also regulate the expression of proteins associated with secretory pathways in the cell [84]. Manipulation of these pathways inevitably changes the viral and host factors that are transferred to surrounding uninfected cells. This could lead to the regulation of key pathways known to be important during HCMV infection such as metabolic activity, apoptosis, immune signaling, and cell cycle regulation. Regulation of these pathways in
uninfected cells could have important implications for virus infection and spread, but the relevance to HCMV infection is largely unknown.

Cells release signaling molecules inside membrane-enclosed compartments termed EVs that transfer proteins and nucleic acids to recipient cells. This signaling mechanism is commonly modified during viral infection. Modulation of the content loaded into EVs during viral infection determines whether these signaling vesicles contribute to host defense or create an advantageous environment for the virus. The transfer of EVs from infected cells can activate innate antiviral activity in uninfected cells to inhibit the spread of the virus [267]. Alternatively, the incorporation of viral genomes or viral proteins into EVs can enhance infection by preventing the antiviral response, or through the escape from neutralizing antibody [282, 284, 285, 302]. Herpesviruses are also known to modulate this signaling pathway, as cells infected with HSV-1 release EVs that inhibit the spread of the virus [270, 271, 303]. EBV and KSHV both package viral miRNAs into EVs that contribute to the tumor microenvironment, and also may enhance virus spread [289-292]. HCMV also incorporates viral proteins into EVs, which may contribute to the transfer of viral antigen to recipient cells [293, 294]. However, the EV contribution to HCMV infection has not been determined. EV biogenesis and release can occur through multiple cellular pathways. One such pathway involves the membrane scission proteins associated with the ESCRT machinery. We and others have reported that ESCRT-III and VPS4 are important for efficient spread of HCMV [223, 304]. Since HCMV reorganizes vesicle trafficking within infected cells and the ESCRTs play a role in infection, we hypothesized that HCMV modulates EVs to enhance virus spread.
In this study, we find that HCMV requires EVs for efficient spread. Inhibition of EV production slowed the spread of HCMV through a monolayer, which could be rescued by addition of purified EVs. We found that infected fibroblasts produce more EVs, which correlates with an increase in the expression of EV biogenesis machinery. EVs produced during infection are morphologically unaltered, but trend to smaller size and contain viral cargo. The functions of the viral components found in EVs are consistent with a role in preparing uninfected cells for the oncoming infection. These results uncover a mechanism by which HCMV modulates a common biological pathway to enhance viral infection.

Results

HCMV regulates expression of EV biogenesis machinery

We and others have previously found that inhibition of the ESCRT proteins hindered the spread of HCMV [223, 304]. The ESCRT proteins promote membrane scission during MVB formation and represent one pathway for the formation of vesicles that can be released as EVs [181]. It is known that inhibition of ESCRT activity through expression of dominant negative ESCRT subunits or RNAi reduces the number of EVs released from the cell [253, 260, 262]. Conversely, expression levels of ESCRT proteins are positively correlated with increased vesicle release [269]. Since ESCRT-III is associated with the scission-promoting function of these complexes, we analyzed expression levels of subunits from this complex during infection of human dermal fibroblasts (HDF). Interestingly, we found that protein levels of ESCRT-III subunits were
increased throughout infection (Figure 3-1A). Transcript levels of the ESCRT-III subunits tested were also increased, indicating that the increase in protein is at least partially due to increased mRNA within infected HDFs (Fig 3-1B). We hypothesized that the increase in ESCRT-III protein levels could lead to an increase in EV production. To test this, we analyzed the intracellular expression levels of common EV surface markers, the tetraspanins CD63 and CD81. We found that intracellular levels of both EV markers were decreased during infection (Fig 3-1C). The decrease in intracellular levels of EV surface markers does not appear to be due to changes in transcript levels as these appear to remain relatively constant throughout infection (Fig 3-1D). The decrease in intracellular EV markers could be explained by an increase in the release of EVs from the cell. To test this possibility, we analyzed extracellular levels of CD63 and CD81 during infection. Interestingly we found that extracellular levels of both proteins increase during infection (Fig 3-1E). This indicates that HCMV infection alters the expression of EV biogenesis proteins and increases the release of EV markers during infection. However, this does not exclude the possibility that a subset of CD63 and CD81 are degraded during infection.

**Inhibition of extracellular vesicle production slows HCMV spread**

The regulation of EV-associated proteins is suggestive of an increase in EV release during infection. In addition to the ESCRT pathway, EV biogenesis occurs through the enrichment of the lipid ceramide at the endosomal membrane.
Figure 3-1: HCMV infection alters EV biogenesis machinery. Uninfected and HCMV-infected (MOI=3) fibroblasts at 24, 48, 72, and 96 hpi were harvested for protein (A and C) and RNA (B and D) analysis. qPCR results are from three independent experiments. Tubulin was used as a loading control for western blot analysis. A) Western blot of the ESCRT-III subunits CHMP6, CHMP4B, and CHMP2B. B) qPCR of CHMP6, CHMP4B, and CHMP2B. *indicates p<0.05 compared to uninfected samples. C) Western blot of EV markers CD63 and CD81. D) qPCR analysis of CD63 and CD81 during high MOI infection. E) Western blot of extracellular CD63 and CD81 released from HCMV-infected fibroblasts.
Sphingomyelinases generate ceramide from a sphingomyelin precursor, and since treatment of cells with nSMase inhibitors prevents EV formation and release from the cell, these inhibitors are commonly used to disrupt EV release during diverse virus infections [265, 267, 268, 305]. To test if EVs generated from this pathway also contribute to HCMV spread, we treated HCMV-infected cells with the nSMase inhibitor GW4869. The efficacy of the inhibitor was confirmed by observing an intracellular accumulation of EV markers (Figure 3-2A). HCMV replication and egress is associated with cellular proteins that are also utilized in the release of EVs [112]. It is possible that in addition to altering EV biogenesis, treatment with GW4869 may also inhibit the replication cycle of the virus. We analyzed intracellular and extracellular virus titers from high MOI infections in the presence of GW4869. Virus replication and egress were unaffected by the block in EV release as virus titers were unchanged in the presence of the inhibitor (Figure 3-2B). GW4869 treatment, even for prolonged periods of time, did not appear to cause toxicity in the fibroblasts because cell viability was similar across all treatment groups (Figure 3-2C). EVs released from infected cells could be transferred to surrounding recipient cells and alter the activity of recipient cells prior to virus infection. Thus, we hypothesized that EVs may have an important effect on the spread of HCMV. To address this, we tested the effect of GW4869 during a low MOI infection. We used a recombinant TB40/E strain that encodes GFP adjacent to the viral immediate early protein 2 separated by the T2A peptide [306]. During infection with this virus, GFP-positive cells can be used to monitor virus spread through the monolayer. In contrast to the high MOI results, the inhibitor slowed the efficiency of virus spread through the monolayer (Figure 3-2D & 3-2E).
Figure 3-2: GW4869 slows HCMV spread. A) Western blot of intracellular CD63 and CD81 in HCMV-infected (MOI=1) fibroblasts at 72 hpi treated with DMSO or 8 μM GW4869. Tubulin was used as a loading control. B) Intracellular and extracellular virus titers from HCMV-infected (MOI=3) fibroblasts at 96 hpi treated with DMSO or 8 μM GW4869. C) Trypan blue viability in uninfected or HCMV-infected (MOI=0.05) fibroblasts at 9 dpi. At indicated time points, cells were trypsinized and resuspended in DMEM before being mixed with trypan blue. Positive control cells were incubated at 65°C for ten minutes. D) Virus spread in HCMV-infected (MOI=0.05) fibroblasts treated with DMSO or 8 μM GW4869. Relative infection measured by GFP expression in the monolayer. E) Fluorescence images from low MOI spread infection in D). Scale bar: 1,000 μm. F) Total cell count of uninfected and HCMV-infected (MOI=0.05) fibroblasts at 9 dpi treated with DMSO or 8μM GW4869. Values in B, C, D, and F are from three independent experiments. * indicates p<0.05.
The reduction in spread following GW4869 treatment was not caused by a reduction in cell density, as total cell counts were not reduced in the treatment group (Figure 3-2F). In fact, cell counts were slightly higher in GW4869-treated cells at 9 dpi. It is well known that HCMV infection causes cell cycle arrest and therefore prevents cell division. Since this is the timepoint with the largest difference in virus spread, we hypothesize that the higher cell count may be caused by an increased number of uninfected cells still undergoing cell division. Therefore, the nSMase inhibitor GW4869 slows HCMV spread without affecting virus egress or cell viability.

**Purification of EVs from HCMV-infected cells**

Our data show that both treatment with the EV inhibitor GW4869 or blocking ESCRT activity slows the efficiency of HCMV spread without inhibiting the viral replication cycle. This data supports the hypothesis that EVs released from HCMV-infected cells are important for virus spread. To gain a better understanding of the role of EVs during infection, we purified EVs from infected or uninfected cells. We chose to purify EVs from HCMV-infected HDFs at 72 hpi since this time point indicated maximal regulation of the EV-associated proteins (Figure 3-1). To characterize the EVs released from infected cells, the vesicles must be separated from viral particles that are also present in the infected culture medium. EVs were separated from viral particles and purified using a combination of high-speed and ultracentrifugation steps with an OptiPrep gradient (Figure 3-3A). Western blot of the isolated fractions for CD63 and CD81 was used to identify fractions that contained EVs, and an antibody to the abundant viral tegument protein pp65 was used to identify fractions enriched with viral particles or dense bodies (Figure 3-3B).
Figure 3-3: EV purification from HCMV-infected cells. A) Protocol to purify EVs from HCMV-infected (MOI=1) fibroblast medium. B) Western blot analysis of the viral protein pp65 and cellular proteins CD63 and CD81 from EV purification. Cell lysate at 72 hpi (Lys), 10,000xg centrifugation pellet (10K), and fractions from the OptiPrep density gradient (1-9). C) Virus titers from a representative EV purification experiment.
CD63 and CD81 positive fractions spanned the 10-30% region of the gradient. Importantly, pp65 was not detected in fractions that were positive for the EV markers, suggesting that EVs were successfully separated from viral particles. To confirm this, we performed an infectious titer assay on all collected fractions. For the majority of the isolations, HCMV was pelleted at a high-speed centrifugation step labeled “10K pellet” as shown in a representative titer assay (Figure 3-3C). In the rare event that infectious virus was present within the gradient, these fractions were discarded and not used for any downstream analysis or experiments. Using this approach, we were able to successfully separate EVs from infectious particles.

**HCMV infection increases EV release**

We next analyzed and compared the EV preps from infected and uninfected cells. EM analysis of the purified EV preps revealed no differences in morphology between the samples (Figure 3-4A). Importantly, we did not observe any viral particles, consistent with our infectious titer assay of vesicle fractions. Cells can release many unique vesicle populations that are characterized by origin of biogenesis, internal cargo, surface markers, and size [259]. Pooled EV fractions were tested for changes in vesicle surface markers by western blot analysis loaded in equal volume (Figure 3-4B). Both infected and uninfected EVs were positive for the vesicle markers CD63, CD81, and Tsg101, and negative for the intracellular marker α-tubulin. Strikingly, the cellular EV markers were more abundant in the EVs purified from infected cells. We hypothesized that an increase in vesicle marker intensity could be occurring through two ways: HCMV infection increases the number of vesicles released from the cell, or infected cells may release vesicles with a higher enrichment of these molecules per vesicle.
Figure 3-4: HCMV infection increases EV production. Analysis of EV populations was carried out using EV fractions from uninfected or HCMV-infected (MOI=1) fibroblasts at 72hpi. A) Electron micrographs of pooled EV fractions. Scale bar: 50 nm. B) Western blot analysis of pooled EV fractions for tubulin, CD63, Tsg101, and CD81 loaded by equal volume. Lysate is from HCMV-infected (MOI=1) fibroblasts at 72 hpi. C) Particle concentration of pooled EV fractions assayed by nanoparticle tracking analysis (NTA). D) Western blot analysis of EV fractions loaded by equal vesicle number. E) Normalized size distribution, mean diameter, and mode diameter of pooled EV fractions. All NTA analysis is from seven independent EV harvests. * indicates p<0.05.
Viral infections are known to increase the release of EVs from infected cells [271, 307]. To address this possibility, we analyzed the vesicle population by nanoparticle tracking analysis (NTA). Interestingly, HCMV-infected cells released three-fold more EVs compared to uninfected cells, suggesting that the increase in extracellular EV markers is a result of increased release of vesicles from the cell (Figure 3-4C). In support of this, western blot analysis of EVs with equal loading of vesicle number showed little difference in EV marker abundance between uninfected and infected samples (Figure 3-4D). In addition to increased vesicle release, HCMV infection also altered the size distribution of the purified EVs. Uninfected cells released vesicles that could be categorized by their diameter into small and large EVs (Figure 3-4E). Interestingly, infected cells released a more uniform population of vesicles that were smaller in size compared to uninfected EVs. This is supported by the fact that infected EVs have smaller median and mode vesicle diameter than uninfected EVs (Figure 3-4E). The trend toward a smaller vesicle size is further indication that HCMV infection alters activity associated with EV pathways. Thus, HCMV-mediated regulation of the EV biogenesis machinery results in increased release of EVs and altered vesicle size.

**EVs from HCMV-infected cells contain viral proteins with late domain sequences**

Our data has shown that EVs released during HCMV infection appear to enhance the efficiency of virus spread. To determine how these EVs contribute to HCMV spread, it is important to identify HCMV-mediated changes in EV cargo. EVs are enriched in a number of signaling molecules, some of which include: lipids, proteins, mRNAs, and noncoding RNAs such as miRNAs. These molecules can have diverse effects on the activity of the recipient cell. The genome of HCMV encodes a number of proteins that
alter the activity of the infected cell to help initiate and establish a productive infection. We hypothesized that HCMV-infected cells transfer these viral factors through EVs to uninfected cells to alter the environment, which helps establish infection and enhance spread. In fact, viral proteins have been found in EVs released from HCMV-infected cells [293, 294]. Upon further analysis of these previous reports, HCMV viral proteins identified in EVs contain amino acid sequences classified as late domains (Figure 3-5A). Late domains were first described as amino acid sequences in viral proteins necessary for a late stage in the viral replication cycle of retroviruses, and are known to be important for protein-protein interactions with ESCRT subunits, serving as a mechanism to recruit the ESCRT machinery during infection [157, 209, 210]. However, late domains can also recruit proteins for incorporation into EVs through interactions with ESCRT proteins [273]. HCMV encodes multiple proteins with predicted late domain sequences even though the ESCRTs are not required for viral envelopment [196, 304]. We analyzed our EV population for the presence of viral proteins (Figure 3-5B & 3-3B). Consistent with previous findings, EVs were positive for the late domain-containing viral glycoprotein gB and negative for the abundant tegument protein pp65, which does not contain a late domain [293, 294]. We also found the UL82 gene product pp71 in the EV population, which also contains a late domain sequence and has a well-documented role in the initiation of infection and manipulation of the antiviral response [53, 54, 72]. Incorporation of HCMV proteins into EVs appears to be specific, as not all late domain-containing proteins (pp150) and proteins without late domains (IE1/2, and UL71) are in EVs [294]. Together, this data suggests that HCMV proteins may be incorporated into EVs through late domain sequences.
Figure 3-5: **EVs from HCMV-infected cells contain viral proteins.** A) Table of late domain sequences within viral proteins detected in infected EVs. B) Western blot analysis of viral proteins in pooled EV fractions from uninfected or HCMV-infected (MOI=1) fibroblasts at 72 hpi.
**Infected EVs do not require CD63 to enhance spread**

In addition to late domains, other mechanisms exist for EV cargo selection. Proteins are selectively incorporated into vesicles through posttranslational modifications or interactions with EV-associated proteins, such as CD63, that target cargo to the site of EV biogenesis [273, 276]. With respect to herpesviruses, CD63 is important for incorporating the EBV latent membrane protein 1 (LMP1) into EVs [272, 274, 275]. Additionally, CD63 is important for the antiviral phenotype of HSV-1 EVs as knockdown of CD63 reduces the antiviral phenotype during HSV-1 infection [270]. Conversely, overexpression of CD63 amplifies the phenotype [271]. To see if CD63 was also important for HCMV spread, we used a lentivirus-encoded shRNA to knockdown expression of CD63 in HDFs (Figure 3-6A). Recent work has suggested that CD63 may be required for HCMV replication in a mechanism that has yet to be determined [308]. However, we found that knockdown of CD63 did not inhibit HCMV replication in a high MOI infection or block virus egress (Figure 3-6B). Surprisingly, knockdown of CD63 also had no effect on the efficiency of HCMV spread (Figure 3-6C). This appears to be in contrast to other herpesvirus infections and may represent a key difference between the mechanisms of EV manipulation. This data indicates that the effector molecules necessary to enhance HCMV spread are incorporated into EVs in a CD63-independent mechanism.
Figure 3-6: Knockdown of CD63 does not inhibit HCMV spread. A) Western blot analysis of CD63 in uninfected fibroblasts transduced with lentivirus expressing shRNA to CD63 or scrambled control. Tubulin was used as a loading control. B) Intracellular and extracellular virus titers from HCMV-infected (MOI=3) transduced fibroblasts at 96 hpi. Titers are from three independent experiments. C) Virus spread in HCMV-infected (MOI=0.05) transduced fibroblasts. Relative infection measured by GFP expression in the monolayer. Values for low MOI spread data represents five independent experiments.
**Extracellular vesicles enhance HCMV infection**

We have shown that HCMV alters the number and composition of EVs released from cells, and that blocking EV biogenesis impairs virus spread. To determine if HCMV-mediated changes in EVs were responsible for enhancing HCMV spread, we tested whether the addition of purified EVs from infected cells could rescue the spread defect caused by the EV inhibitor GW4869. Under physiological conditions, the number and frequency of vesicles that individual cells receive from the extracellular environment is unknown. However, using a similar approach as was used to study EVs in other herpesvirus infections, we tested whether the transfer of purified EVs could rescue the spread defect of GW4869 treatment [270, 291] (Figure 3-7A). To alleviate the concern that we may have small amounts of undetectable virus in our purified EV population, all EV isolations were performed on cells infected with a TB40/E virus that does not express any fluorescent proteins. Even if a small amount of contaminating virus is present in fractions, this would not impact the analysis of our spread experiment, which depends upon the presence of GFP to monitor virus spread. We found that treatment of cells with EVs purified from infected HDFs enhanced the spread of HCMV in the presence of the EV inhibitor (Figure 3-7B). The degree of rescue was variable amongst the experiments and this could be due to the quality of the purified EVs. Although addition of EVs resulted in an increase in virus spread, spread was still reduced compared to the untreated sample (Figure 3-7C). This may be due to the number of vesicles added, frequency of vesicle addition, or when the purified vesicles were harvested. Interestingly, EVs purified from GW4869-treated cells were unable to rescue the spread phenotype of the inhibitor (Figure 3-7D).
Figure 3-7: EVs from HCMV-infected cells enhance virus spread. A) Experimental outline to transfer purified EVs to HCMV-infected (MOI=0.05) fibroblasts. 3,000-5,000 purified EVs were added to spread experiments at 3, 4, and 5 dpi as indicated and GFP expression was measured at 3, 6, 9, 12, and 15 dpi. B) Fold increase for four individual experiments showing virus spread relative to GW4869-treated HCMV-infected fibroblasts. C) Fluorescence images from the spread infection in B). Scale bar: 250 μm. D) Virus spread in fibroblasts treated with 8 μM GW4869 where EVs purified from cells treated with 8 μM GW4869 were added. * indicates p<0.05 compared to untreated control. E) Virus spread in the presence of EVs purified from HCMV infection. * indicates p<0.05.
This is further indication that treatment with GW4869 disrupts the EV production during HCMV infection. Since EVs enhanced HCMV spread in the presence of the GW4869, we next tested whether EVs could further increase the spread of an untreated infection. We found that the addition of EVs to an untreated HCMV infection further enhanced the efficiency of spread (Figure 3-7E). This data clearly indicates that extracellular vesicles released from HCMV-infected cells transfer signals to uninfected cells that are important for efficient virus spread.
Within an infected cell, HCMV extensively regulates key pathways to enhance viral replication. Elucidating the mechanisms involved in viral manipulation of cellular machinery will lead to a better understanding of HCMV infection and guide new therapeutic approaches to reduce disease burden. An important hallmark of HCMV infection is membrane remodeling within the cell, which contributes to efficient viral replication. Morphological changes to cellular membrane have been associated with HCMV since the initial characterization of HCMV-infected cells as being enlarged with cytoplasmic inclusion bodies [8]. A consequence of altered membrane organization is differential vesicular trafficking within the cell, which has important implications for the intracellular transfer of molecules during infection. Infected cells upregulate mRNA levels of cellular genes associated with increased activity of secretory vesicle pathways [110]. This suggests that HCMV modulates communication between infected cells and the surrounding uninfected cells, but the impact on infection and spread is largely undetermined.

To gain a better understanding of the regulation of vesicular trafficking during HCMV infection, it is important to define the role of proteins associated with these pathways. One group of proteins that is important for the formation of vesicles is the ESCRT machinery. The ESCRTs are necessary for membrane scission events within the cell and they are required by many enveloped viruses during envelopment. The focus of this work was to investigate the role of the ESCRT proteins during HCMV infection. Given the well-defined role of the ESCRTs during virus budding, we began by testing the hypothesis that HCMV requires the late ESCRTs for envelopment.
To our surprise, we found enveloped virions, both intracellular and extracellular, in cells where the ESCRT activity was blocked. This disproved our hypothesis that HCMV requires ESCRT-III and VPS4 for production of infectious virions. While we were unable to find a role for ESCRT-III in HCMV envelopment, we did find that efficient spread of the virus was reduced in the absence of functional ESCRT-III subunits. Based on this observation, we hypothesized that the ESCRTs were needed to produce EVs that transfer signals to neighboring cells to prepare them for the incoming infection. In support of this hypothesis, we found that an EV inhibitor also reduced HCMV spread. Importantly, the addition of EVs purified from HCMV-infected cells enhanced the spread of HCMV infection in GW4869-treated or untreated fibroblasts. This collection of data supports a role for the ESCRTs during HCMV infection through the production of EVs that help enhance the efficiency of virus spread. The data generated to test these hypotheses has led to a new model of the activity of ESCRT-III and EVs during HCMV infection and spread (Figure 4-1). Important findings of this work and new directions that will help build upon this model are discussed below.
1. HCMV alters EV biogenesis in infected cells

2. EVs transfer proviral factors to uninfected cells

3. HCMV spread is enhanced by proviral EVs

Figure 4-1: Model of modulation of EV biogenesis machinery to enhance HCMV spread. 1) During HCMV replication, ESCRT-III subunits are upregulated and infected cells increase the release of EVs. 2) EVs are transferred to uninfected cells and alter the activity within uninfected cells that make them more conducive to HCMV infection. 3) This altered environment enhances the efficiency of HCMV replication and spread.
Differential regulation of ESCRT proteins during HCMV infection

Localization of ESCRT-III and VPS4 during HCMV infection

Proteins involved in virus assembly and envelopment localize to the cVAC during the late stages of infection. We tested the localization patterns of ESCRT proteins during infection to gain insight into the function of the proteins during HCMV infection. Others have shown that VPS4, and the accessory factors associated with VPS4, localize to the cVAC [90, 223, 309]. VPS4 accumulation at the cVAC is consistent with the hypothesis that the ESCRTs are involved in envelopment. However, our analysis of ESCRT-III subunit localization found that CHMP6 was the only ESCRT-III protein tested that accumulated within the cVAC. The differential localization of VPS4 and ESCRT-III subunits is interesting, but the lack of ESCRT-III subunits in the cVAC did not support our envelopment hypothesis.

While CHMP6 accumulated in the cVAC, this is not likely to be through a canonical ESCRT recruitment mechanism. CHMP6 is the only ESCRT-III subunit that is modified through myristoylation of the N-terminal glycine, and mutations at this site prevent membrane localization of the protein [156, 310]. Rather than recruitment by upstream ESCRTs or viral proteins, CHMP6 myristoylation may cause the protein to accumulate in the cVAC where there is an enrichment of membrane. In support of this, expression of CHMP6 with an N-terminal GFP tag, which should prevent myristoylation, did not show cVAC enrichment (Appendix A). If CHMP6 is only transiently associated with membrane through myristoylation, then this would explain why the other ESCRT-III subunits are not recruited to the cVAC by CHMP6.
It is also noteworthy that VPS4 accumulates in the cVAC, which is clearly not dependent on recruitment by ESCRT-III subunits. VPS4 is an ATPase, and this activity could be utilized outside of the ESCRT machinery. In other viral infections, viral proteins can directly interact with VPS4 without requiring recruitment through ESCRT-III subunits [204]. It is possible that an HCMV viral protein recruits VPS4 to the cVAC for an ESCRT-independent function. ESCRT-III subunits contain MIM sequences that interact with the MIT motif of VPS4 to facilitate recruitment of VPS4 to various sites within the cell [184-186]. Interestingly, the viral protein UL71 contains a sequence similar to MIMs found in ESCRT-III subunits. In vitro analysis found that UL71 interacts with VPS4 through the MIM-like motif, and VPS4 was no longer recruited to the cVAC during a viral infection with a recombinant virus lacking the MIM-like sequence in UL71 [311]. While UL71 is important for the recruitment of VPS4 to the cVAC, there was no defect in HCMV envelopment and replication when using the recombinant virus [312].

The finding that VPS4 recruitment to the cVAC is not required during HCMV infection supports data presented in this work, which found that inhibition of VPS4 did not block viral replication in fibroblasts. However, this does not rule out the possibility that VPS4 interacts with viral proteins such as UL71 and has a cell type or context-dependent function during HCMV infection that was not identified by the conditions used in these studies. Further analysis of the importance of VPS4 during infection of other cell types may help clarify this.
ESCRT-III upregulation during HCMV infection

Our investigation of the ESCRTs also uncovered upregulation of ESCRT-III expression at the level of transcript and protein during HCMV infection. Little is known about transcriptional regulation of ESCRT-III subunits, making it difficult to speculate how this is occurring during infection. However, modulation of ESCRT protein expression occurs in other viral infections as well, and may be a common mechanism to alter the activity of this machinery. VPS4 is downregulated during HSV-1 infection of neuronal cells, which may reduce the activity of this pathway [313]. Conversely, the upregulation of ESCRT proteins during HCMV infection could result in an increase in ESCRT activity during infection. We speculate that increased ESCRT-III levels could at least partially explain the observed changes in EV production and size, which will be further discussed in a latter section. However, an increase in ESCRT levels could have additional effects within the infected cell. There is some data to suggest that the ESCRTs are responsible for the lysosomal degradation of HCMV capsids through the MVB pathway. Previous work found that blocking the ESCRT pathway with siRNA increased levels of extracellular virus released from cells [196]. This same study found that treatment of infected cells with protease inhibitors also increased extracellular virus titers, indicating that the ESCRT machinery may represent a mechanism to reduce the amount of virus released from cells. In support of this, recombinant viruses null for the expression of viral proteins required for envelopment accumulate capsids and viral tegument proteins in MVB-like structures [106]. While not statistically significant, we also found a trend toward increased extracellular virus titers produced from cells expressing dominant negative ESCRT proteins. These data indicate that there could be a
balance between targeting of capsids to membrane for envelopment and aberrant budding into MVBs that results in degradation of the virus. The increase in ESCRT protein expression in the cell could be a cellular response to control the amount of virus produced during infection. The differential localization of the ESCRT proteins may also be an attempt by the virus to reduce capsid degradation through this pathway by preventing accumulation of the ESCRTs within the cVAC. However, this does not seem to be a major pathway of virus control since we and others have shown that virus spread is enhanced by ESCRT machinery [223, 304].

**Cytotoxic effects of blocking ESCRT activity**

The function of the ESCRT machinery is essential for the viability of all cells. For this reason, attempts to generate cell lines and animal models deficient in ESCRT proteins have had little success [200, 201]. However, toxicity caused by a block in ESCRT activity will not always be as apparent as cell death. It is well documented that HIV requires the ESCRTs for virus budding [157]. However, extensive work on HIV and the ESCRTs identified a number of conditions where a block in ESCRT activity reduced infectious virus production in an envelopment-independent manner [198]. In these conditions, expression of the viral protein Gag was severely reduced within infected cells, due to cell toxicity caused by the block in ESCRT activity. Cytotoxicity caused by blocking the ESCRT pathway may be difficult to detect during infection with HCMV, which encodes numerous viral factors that prevent activation of apoptotic pathways [314]. For this reason, we used multiple assays to assess the viability and metabolic status
of our cells during conditions where the ESCRT pathway was blocked. To be sure that a reduction in cell viability was not masked by the fact that HCMV may modulate downstream factors measured by our assays, we also assessed the establishment of HCMV infection by measuring abundance of viral protein expression within infected cells.

This approach was crucial to our ability to appropriately interpret the results of our study. Our first attempt to investigate the role of the ESCRTs during HCMV infection utilized expression of dominant negative ESCRT-III subunits from the genome of a recombinant virus. This resulted in robust expression of dominant negative ESCRT-III proteins and a severe reduction in infectious virus produced in these cells. Had it not been for thorough testing for signs of cytotoxicity, the results of this study would have been interpreted incorrectly. Our second approach utilized lentiviral transduction of inducible dominant negative ESCRT constructs that allowed more controlled expression of the proteins. This approach was still sufficient to block ESCRT activity as measured by receptor degradation and inhibition of HSV-1 replication. This strategy allowed us to properly study the role of the ESCRTs during HCMV infection. We hope that the results of this work will set a new standard for future ESCRT studies and testing for cytotoxicity.
HCMV Envelopment

Viral proteins as functional homologs of ESCRT-III subunits

In an attempt to define the mechanism of HCMV envelopment, we tested the contribution of ESCRT-III and VPS4 during infection. To our surprise, we found no evidence that HCMV requires the ESCRTs for production of infectious virions. This finding leads to an important question: how does HCMV facilitate viral envelopment using an ESCRT-independent mechanism? ESCRT-independent envelopment is not without precedent, as both influenza virus and SFV bud by mechanisms that do not require this machinery [224, 225]. In the case of influenza virus infection, the viral protein M2 mediates membrane scission and envelopment. While M2 does not have sequence homology to any ESCRT protein, M2 has many functional characteristics that allow it to have similar membrane scission activity. M2 is an integral membrane protein, and membrane association of some kind is a requirement for a protein that must deform membrane [315]. Oligomerization is key for ESCRT-III to constrict and deform membrane during the scission process. M2 also forms oligomers in vitro, which may allow M2 to perform similar functions as ESCRT-III filaments [316]. Finally, and most importantly, ESCRT-III and M2 are both capable of membrane scission using in vitro GUV experiments [224].

One possibility is that HCMV encodes a protein that, like influenza virus M2, is functionally homologous to the ESCRT proteins. HCMV UL71 is a viral protein that has experimental evidence of some of these characteristics. UL71 is known to be important at the late stages of envelopment, as infection with a UL71 null virus results in capsids that
are trapped in stalled unscissioned membrane buds [106]. UL71 is also conserved among other herpesviruses, and these proteins also have roles in viral envelopment, albeit not to the same extent as UL71 [298, 317]. UL71 is associated with membrane within the cell and colocalizes with markers of the TGN [108]. The HSV-1 homolog UL51 is also membrane-associated and this is dependent on palmitoylation of UL51 [318, 319]. Sequence alignment of HSV-1 UL51 and HCMV UL71 predicts that the palmitoylation site is conserved, although this has not been validated [319]. In addition to membrane association, UL71 is also known to form oligomers which are necessary for HCMV envelopment [107]. These characteristics give support to the idea that UL71 may act as a functional homolog to the ESCRT proteins. This would provide a mechanism for HCMV envelopment that is independent of the ESCRT pathway. However, evidence that UL71 or any associated viral proteins are directly capable of membrane scission is still lacking. In vitro analysis of UL71 is required to address this.

**HCMV envelopment through other cellular proteins**

While viral proteins may mediate envelopment, an alternative possibility is that HCMV utilizes other cellular machinery to facilitate envelopment. Membrane scission through the ESCRT pathway is thought to be mediated by force generated by the AAA ATPase VPS4 [190]. While VPS4 is not required for envelopment, HCMV may recruit other ATPases to facilitate membrane scission. Spastin is an ATPase that is associated with the ESCRT machinery [320, 321]. In fact, Spastin is required for ESCRT-mediated scission of membrane and microtubules during abscission [322]. The localization and activity of spastin during HCMV infection has not been explored. If HCMV could recruit spastin to sites of envelopment, this could provide a potential mechanism for membrane
scission. It can be assumed that spastin, like VPS4, would not be sufficient to drive
t membrane scission without other factors. This possibility would require other viral or
cellular proteins to contribute to the membrane constriction process prior to force
generation by the ATPase.

Proteins associated with autophagy are also utilized for viral envelopment. Cells
infected with HBV have increased autophagy and blocking this activity with inhibitors or
RNAi reduced viral replication at an envelopment step [323]. The role of autophagy
during HCMV replication is highly complex. HCMV infection is known to temporally
regulate autophagic flux in multiple cell types [324, 325]. Autophagy was thought to be
inhibitory to HCMV infection, because treating cells with compounds that activate
autophagy inhibits viral replication [325]. However, it was recently reported that HCMV
viral replication is inhibited by knockdown of proteins associated with autophagy, and
proteins associated with autophagic membranes were also found in purified viral particles
[326]. It is possible that HCMV utilizes proteins associated with autophagy for
membrane deformation during envelopment. The ESCRT machinery has recently been
identified to be important for the closure of autophagic membrane during formation of
the phagophore [327, 328]. VPS37A is one of the ESCRT proteins identified to be
important for closure of the autophagic membrane, and the role this ESCRT subunit
during HCMV infection has not been explored. It is possible that the autophagy pathway
and select ESCRT proteins not yet tested during infection are utilized for membrane
scission during HCMV envelopment.
**HCMV modulates EV morphology and composition**

**HCMV infection alters EV size distribution**

Our work uncovered significant regulation of EV biogenesis machinery during HCMV infection. This caused HCMV-infected cells to release three-fold more vesicles than uninfected cells. EVs released from HCMV-infected cells also had an enrichment of smaller vesicles within the total vesicle population. Of the diverse population of EVs released from cells, exosomes are a subset of vesicles generated from endosomal membrane and characterized by a diameter of 50-150 nanometers [259]. The ESCRT proteins contribute to the formation of exosomes, and the increase in ESCRT protein levels during infection could be responsible for the shift in size toward the exosome population [260, 329]. However, further investigation is needed to explain this. Size distribution analysis of EVs purified from HCMV-infected cells with a block in ESCRT activity would begin to address this possibility. If infected cells with a block in ESCRT activity no longer have a difference in size distribution compared to uninfected cells, then the increased expression of ESCRTs is likely the cause of this shift.

The ceramide-dependent vesicle biogenesis pathway also generates EVs within this same size range [265]. The enzymes required for ceramide production are SMases, and increased expression of SMase in cells is also associated with an increase in EV production [265, 266, 305]. Our work does not investigate the expression kinetics or activity of SMases during HCMV infection. However, we did uncover that inhibitors of these enzymes reduce virus spread, suggesting that the levels of SMases during HCMV infection may be an important factor in the differences in vesicle characteristics reported
in this work. This must be investigated along with the contribution of the ESCRTs to understand what contributes to changes in vesicle size and production during HCMV infection. This may also provide insight into selective packaging of cargo during infection, since these pathways may generate vesicles with different signaling molecules.

Selective incorporation of viral factors into EVs

In addition to changes in size distribution, we found that HCMV infection alters EV composition. Several viral proteins are incorporated into EVs, and these proteins interestingly all contain late domain sequences [293, 294]. We used this information to attempt to identify new viral proteins within these vesicles. Using this approach, we identified that the viral protein pp71 is also present in EVs released from infected cells. The fact that all viral proteins that have been found in EVs contain late domains suggests that this is a mechanism that the virus utilizes to incorporate viral proteins into vesicles. However, not all of the viral proteins with late domains that we tested (UL48 and pp150) were found in EVs. While unexpected, this finding does not refute the idea that viral proteins are incorporated through interactions with ESCRT proteins. Late domains are small sequences of just a few amino acids. The small sequence and the fact that variability is tolerated at certain residues (i.e. YxxL) means that these motifs are commonly found in many proteins. While not all of the proteins with late domains were incorporated, no study has ever tested whether these proteins actually interact with ESCRTs through their predicted late domain sequences. These interaction studies need to be done to fully understand the significance of these sequences and their role on EV incorporation. As discussed in the introduction, other mechanisms for selective protein incorporation through protein-protein interactions and posttranslational modifications
exist [274-276]. These routes of selective incorporation may facilitate the packaging of other viral proteins into EVs. The contribution of these other mechanisms must be addressed in future studies.

A key component of EVs that is not addressed in this study is the impact of miRNAs on virus spread. EBV, KSHV, and HSV-1 all package viral miRNAs into EVs [288-290, 303]. Incorporation of miRNAs into EVs is a selective process mediated by RNA binding proteins that interact with specific sequences in miRNAs [279-281]. These RNA binding proteins are normally found in the nucleus of cells, but cytoplasmic translocation of the proteins is required for incorporation of miRNAs into EVs. Two of these RNA binding proteins, hnRNPA2B1 and SYNCRIp, are upregulated during HCMV infection and the proteins accumulate in the cytoplasm during the course of infection (Appendix B). This cytoplasmic accumulation could result in an increase in selective incorporation of miRNAs into EVs during infection, but cytoplasmic accumulation of hnRNPA2B1 also occurs during HSV-1 infection as part an antiviral response that contributes to control of viral infection [330].

It was recently shown that HCMV IE86 interacts with hnRNPA2B1 and SYNCRIp [331]. Since IE86 is found in the nucleus and cytoplasm during infection, these interactions could regulate the nuclear to cytoplasmic translocation of the RNA binding proteins and affect the incorporation of HCMV miRNAs into EVs. Interestingly, miRNAs encoded by HCMV contain potential binding sequences for these RNA binding proteins and these miRNAs target pathways involving immune signaling, apoptosis, and cell cycle [64, 67-71, 332, 333] (Appendix B). While we do not report detection of the miRNAs in EVs, there is evidence that packaging of HCMV miRNAs occurs during
infection. Recently, HCMV miRNAs were found in EVs purified from infected newborns, and their presence is correlated with the severity of disease in the newborn [295]. Incorporation of HCMV miRNAs into EVs provides the virus with a means of modulating the activity of uninfected cells, a phenomenon that has been largely unexplored.

The role of HCMV glycoproteins on the surface of EVs

HCMV viral glycoproteins have been found on EVs purified from infected cells in all studies of HCMV and EVs [293, 294]. This finding has the potential to impact how these vesicles function in different environments. This may affect the type of cell EVs enter and the mechanism of entry into recipient cells. EV entry is highly dependent on the surface proteins present on the vesicle and recipient cell. Because of this, entry of different vesicles can occur through fusion at the plasma membrane or through receptor mediated endocytosis [334]. EV targeting and entry into recipient cells is similar to the concept of viral tropism in that they can only enter cells that have cell surface receptors complimentary to what is present on the surface of the EV. The presence of the viral glycoproteins on the surface of the EVs released from infected cells may influence the “tropism” of the EVs. EVs that contain the surface glycoproteins may also be capable of entering any cell that HCMV can enter. This may be an important way to ensure that the proviral effect of the EVs is transferred to any cell that the virus may encounter. At this point, glycoproteins have only been identified in isolation, and we have no evidence that they are in the proper glycoprotein complexes required to facilitate attachment and entry. This must to be addressed to better understand the role of the HCMV glycoproteins on EV entry.
Viral glycoproteins on the surface of the EVs also have important implications for the antibody response in vivo, because the glycoproteins represent major epitopes of the neutralizing antibody response. If the HCMV glycoproteins facilitate EV entry, then antibody response may also bind and neutralize the proviral effects of the EVs. In this scenario, the activity of the EVs may be limited by an antibody response. However, the EVs could also be important decoys to limit the amount of free antibody in the environment, in a similar way that dense bodies are thought to influence the immune response. As more is understood about the EVs and their role in HCMV infection, it will be important to understand how HCMV neutralizing antibodies affect the function of the EVs.

**Potential effects of EVs on recipient cells**

The incorporation of viral proteins into EVs allows viruses to regulate the activity of uninfected cells even in the absence of infection. This work identifies that the viral protein pp71 is packaged into EVs, and provides a potential mechanism for HCMV miRNA incorporation. The role of pp71 during the early stages of infection initiation is well documented [53, 54, 72]. In fact, transfection of plasmids encoding pp71 into cells prior to infection makes HCMV DNA more infectious [53]. The HCMV miRNAs that contain potential binding sequences target pathways involved in immune signaling, apoptosis, and cell cycle arrest [64, 67-71, 332, 333]. Together the presence of these viral factors, both protein and miRNAs, would have clear effects on the activity of recipient cells. The fact that EVs released from infected cells enhance HCMV spread led us to hypothesize that these viral factors are transferred to uninfected cells to establish a proviral environment that enhances infection efficiency.
While EVs enhance spread, the data from this work do not identify the function associated with the EVs that contributes to HCMV spread. Future studies will need to analyze the effects that EVs have on recipient cells to better understand how they contribute to infection. The presence of the viral factors above provides straightforward targets to test, such as reduced immune signaling, inhibition of apoptosis, or cell cycle arrest in cells that receive EVs. While these should be directly tested, the redundancy in the pathways targeted by different HCMV miRNAs may mean that loss of a single miRNA may not reduce the spread of the virus. Additionally, testing the role of individual viral factors is a very narrow approach based on the expected activity of a few out of the likely hundreds of still unknown proteins and miRNAs that are present in the vesicles. The effect the EVs have on recipient cells will be the result of the combination of viral and cellular factors that are transferred through these vesicles. Analysis of these effects at a larger scale, such as transcriptomic, proteomic, or metabolomic analysis, will provide a more comprehensive understanding of the contribution of the vesicles during HCMV spread.

**ESCRT-dependent and ESCRT-independent EVs enhance HCMV spread**

**ESCRT and ceramide-dependent biogenesis**

We found that inhibition of ESCRT activity slowed HCMV spread. Since this was not caused by a defect in viral envelopment, we hypothesized that HCMV may modulate EVs released from infected cell to enhance spread. To test this, we inhibited EV production and release through ceramide-dependent mechanisms to see if this slowed
virus spread. We found that a block in ESCRT-independent EV production with the nSMase inhibitor GW4869 also slowed HCMV spread. The ESCRTs and ceramide accumulation are thought to represent independent pathways of EV biogenesis, and in some cases, these pathways may even generate vesicles with distinct cargo [265, 335, 336]. This can be at least partially explained by mechanisms of selective packaging. For example, proteins that are incorporated into EVs through late domains would be expected to be found in ESCRT-dependent EVs. The finding that ESCRT-dependent and ESCRT-independent EVs have similar effects during a viral infection has been previously described [267]. This does however raise an important question: what is the functional contribution of each of these EV pathways during HCMV infection?

Work to extensively characterize differences in size, membrane composition, and protein surface markers of EVs from different biogenesis pathways has led to a comprehensive list of characterization criteria for vesicle populations [259]. However, the field is still technically limited in the ability to separate these vesicles from a heterogenous population of EVs harvested from cells. Future work can begin to address this through purification of vesicles from cells where either dominant negative ESCRT expression or GW4869 treatment was used to block one pathway. These purified vesicles can be analyzed for differences in composition and function when transferred to recipient cells. It would be expected that the EVs from different pathways will have a combination of shared and distinct cargo within their vesicles. This approach can begin to assess whether it is a common molecule found in both populations that is responsible for enhancing virus spread, or if each vesicle carries distinct molecules to recipient cells that contribute to the phenotype. To address this, spread experiments where EVs purified
from dominant negative ESCRT or GW4869-treated cells can be added back to spread experiments to see if they can rescue the spread phenotype caused by one or both of these conditions.

**CD63-dependent biogenesis**

An important finding of this work was that CD63-dependent biogenesis of vesicles was not required for HCMV spread efficiency. This is a unique finding compared to other herpesviruses, which modulate EVs in CD63-dependent pathways. The interaction between the EBV protein LMP1 and CD63 facilitates LMP1 incorporation into EVs, and this interaction also increased secretion of vesicles [274]. EVs released from HSV-1 infected cells induce antiviral signaling pathways in recipient cells that control virus spread, and they are generated in a CD63-dependent manner. Overexpression of CD63 in HSV-1 infected cells increases vesicle release and amplifies this phenotype, whereas knockdown of CD63 reverses the phenotype [270, 271]. In our work with HCMV infection, CD63 was present on the EVs that were released from infected cells. Given this information, it was surprising that knockdown of CD63 did not reduce HCMV spread.

While unexpected, this result provides insight into cargo packaging during HCMV infection. During EBV infection, CD63 is important for the changes in metabolic signaling induced by EVs because it is responsible for incorporating LMP1, the protein responsible for these metabolic changes, into the vesicle [274]. Currently, we only know of a few proteins that are incorporated into EVs released from HCMV infected cells. Unlike late domains, CD63-interacting partners do not have a well-described motif that could be used to predict potential viral proteins incorporated into the vesicle through
CD63. While there may be HCMV proteins incorporated into EVs through interactions with CD63, our data suggests that these are not required to enhance virus spread in our system.

The results from the experiments testing the role of CD63 during virus spread have important implications about EV biogenesis during HCMV infection. Autophagic flux is an important regulator of vesicle release, and recent data has shown that CD63 is a regulator of autophagy. CD63 inhibits autophagy, which results in the increased release of EVs [272]. This data suggests that the increase in EV release during CD63 overexpression is due to inhibition of autophagy. Conversely, knockdown of CD63 will result in increased autophagy in cells and reduce the number of vesicles released.

Autophagy is regulated during HCMV infection and viral proteins inhibit autophagic flux during infection [325, 337]. Since HCMV already heavily modulates autophagy, knockdown of CD63 in HCMV-infected cells may not result in the expected increase in autophagy, and therefore not cause a decrease in vesicle release. While we had a strong reduction of CD63 in our knockdown cells, we did not measure changes in EV release from these cells. It is possible that EV levels would be unaffected by CD63 knockdown because of the effects that HCMV has on autophagy. This would mean that CD63 would not be an important regulator of EV release in any HCMV infected cell.
The Role of EVs in vivo

This work is the first to show that EVs enhance HCMV spread through a fibroblast monolayer. However, the content and function of EVs is likely to change with the context of infection within a host. It is known that the same EVs transferred to two different cell types can cause unique effects on each cell. During HCV infection, EVs transfer infectious RNA and enhance spread of the virus in the presence of neutralizing antibody in the Huh7.5 hepatoma cell line [282]. However, EVs transferred to plasmacytoid dendritic cells activate innate immune signaling that slows the spread of infection [267]. We found that EVs released from HCMV-infected cells are important for virus spread in fibroblasts. However, it does not rule out the possibility that EVs are important during other stages of HCMV infection. In fact, it is already known that EVs released from HCMV-infected endothelial cells can activate antiviral activity of T cells [293]. The same study also reported that EVs released from infected fibroblasts were not able to activate the T cells in the same way. The role of EVs during virus spread within endothelial cells was not addressed in this study.

Within a host, HCMV infects a variety of cell types and can undergo lytic and latent stages of viral infection. This study provides a system to begin to understand how HCMV regulates EV pathways, but it will be important to understand the functional importance of EVs during infection of different cell types and during latent infection. This can be addressed by attempting similar studies in other cell types, such as endothelial, epithelial, and myeloid cells that HCMV is able to infect under cell culture conditions. However, this still does not address the fact that EVs from a single infected
cell will be transferred to numerous cell types during an in vivo setting. Some initial data suggests that HCMV miRNAs are found in exosomes from human samples and that this correlates with infection severity [295]. This provides strong support to this work, but in vivo models of infection must be used to fully address the role of the EVs during HCMV infection.

**Final Remarks**

In summary, the objective of this work was to determine the importance of the ESCRT machinery during HCMV infection. We found that HCMV, like many other viruses, utilizes the ESCRT machinery during infection. However, the ESCRTs were not required during HCMV envelopment. Instead we found that the ESCRTs were important for efficient HCMV spread through the formation of proviral EVs released by HCMV-infected cells. While the majority our knowledge of the biology of HCMV infection is focused on the infected cell, this data suggests a more global regulation of the activity of uninfected cells to facilitate successful spread of HCMV. This also generates a foundation for further exploration into the role of EVs in virus spread and pathogenesis.
Chapter 5: Materials and Methods
**Cell culture.** Normal human dermal fibroblasts (Cell Applications Inc. 106-05n and Sigma-Aldrich 106-05N), normal human lung fibroblasts, MRC-5 cells (ATCC CCL-171), and HEK-293TN (provided by David Spector) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Corning) containing 10% fetal bovine serum, 2mM GlutaMAX (Gibco) 100 U/mL penicillin and 100 g/mL streptomycin (Corning). Cells were maintained at 37°C with 5% CO₂.

**BAC mutagenesis.** Recombinant viruses were generated using BAC mutagenesis in E. coli strain SW105 and galK selection as described previously [338]. Inducible ESCRT constructs were generated with pTRE3G-Bi and pEF1a-Tet3G (Clontech). ESCRT-III subunits C-terminally tagged with GFP in pEGFPN2 (Clontech) were inserted into pTRE3G-Bi using restriction enzyme digest with BglII and NotI. Recombinant viruses were made by PCR amplification of the tet-inducible promoter and GFP-tagged subunits and recombination into the EF1a-Tet3G AD169 BAC which has been described previously [86]. The EF1a-Tet3G construct was inserted near the TRS1 region of the genome. AD169 encoding EF1-Tet3G is used as the parental control virus for all recombinant viruses expressing dominant-negative ESCRT-III subunits. The pTRE3G-Bi promoter construct was inserted near the US34a region. The following primers were used: FOR

5’-AGGTGGCGAGGTGTGAGGATGAAACATATGCAGATACGCAGTGTTGTTAAAGTGCCACCTGACGTCG, REV

5’-GACTTTCTACACTGAAAGTGCGTTGTACGATTACACGGGTTTCTCGGAGTGAGCGAGGAAGCTCGG

BAC insertion sites were PCR amplified from purified
BAC DNA and verified by Sanger sequencing (GENEWIZ). TB40/E IE-2A-GFP was generated as previously described [306].

**HCMV infections and virus titrations.** TB40/E, AD169, and derivatives listed above were generated from BAC stocks. To generate virus stocks, purified BAC DNA was electroporated into MRC5 cells according to previously published protocols [339]. Infected cells were used to infect MRC5 cells in roller bottles (Greiner) to produce larger stocks of virus for experimental infections. Virions produced in roller bottles were concentrated by ultracentrifugation on a 20% sorbitol cushion at 20,000 rpm, for 1 hour at 20°C in a Beckman SW32 rotor.

HCMV infections were done at an MOI of 0.05 for multi-step growth curves and MOI 3 for single-step growth curves. Cell counts were determined using TC20 Automated Cell Counter (BioRad). Virus infections were incubated for 3 hours at 37°C before replacing with fresh media. For inducible ESCRT infections, 100 ng/mL doxycycline (Enzo Life Sciences) was added to the media at indicated time points. TB40/E IE-2A-GFP was used for virus spread experiments. Unfixed HDFs in 24-well plates were imaged at the indicated timepoints to calculate the total area of GFP positive pixels within each image. Spread values are relative to 15 dpi control samples. Images were acquired on C2+ Confocal Microscope System (Nikon) and analyzed using Nikon Imaging System Advanced Research Analysis Software.

For virus titers, cells and media were harvested separately to collect intracellular and extracellular virus. Cells were scraped into cell culture medium, sonicated 10 times with 1 second pulses, vortexed for 15 seconds, and centrifuged at 13,000 rpm for 10 min.
Virus was collected and flash-frozen in liquid nitrogen before storage at -80°C. HCMV virus stocks and samples for growth curves were titrated by serial dilutions on MRC5 cells and quantified by the immunological detection of immediate-early proteins as previously described [340]. Either a rabbit polyclonal antibody (a kind gift from Jim Alwine [341]) or a mouse monoclonal (clone D4 generated by Neil Christensen [342]) that detect exons 2 & 3 of the HCMV major immediate early proteins were used. Images of stained monolayers were acquired on a Nikon Eclipse Ti Inverted microscope and fluorescent nuclei were quantified using the NIS Elements Software.

**HSV infections.** Transduced and selected HDFs (as described in next section) were infected with the KOS strain of HSV-1 at an MOI of 5 for 1 hour at 37°C. This MOI was calculated using the viral titer that was previously determined by plaque assay on Vero cells. After 1 hour of infection, HDF cells were rinsed with PBS and then incubated with regular infection media with or without doxycycline at 37°C. At 24 hours post infection when CPE was apparent, HDF cells and media were harvested together and placed through 3 freeze/thaw cycles prior to titration. Viral titer was determined for each sample by plaque assay on Vero cells.

**Lentivirus production and cell selection.** Lentivirus was made using the 3rd generation packaging system: pMDLg/pRRE and pRSV Rev were a gift from Didier Trono (Addgene plasmid # 12251 and 12253), and pCMV-VSV-G was a gift from Bob Weinberg (Addgene plasmid # 8454). pCDH vector as well as VPS4A WT and E228Q were a gift from J. Lammerding. To make inducible ESCRT constructs in the pCDH
vector, we replaced the CMV promoter of pCDH with a tet-inducible promoter to make inducible pCDH (pCDHi). The pCDH CMV promoter was removed by restriction enzyme digest with SpeI and NotI. The tet-inducible promoter was removed from pTRE3G-Bi by digestion with XbaI and NotI and ligated into pCDH with compatible overhangs between SpeI and XbaI. CHMP-GFP sequences were inserted into pCDHi by PCR amplification from pEGFPN2 and restriction enzyme digestion using BamHI and NotI using the following primers:

CHMP6-FOR 5’-GCATGGATCCATGGGTAACCTGTTCGGCCG
CHMP4B-FOR 5’-GCATGGATCCATGTCGGTGTTCGGGAAGCTG CHMP4C-FOR 5’-GCATGGATCCATGAGCAAGTTGGGCAAGTTC CHMP3-FOR 5’-GCATGGATCCATGGGCTGTTTGGAAAGAC CHMP2A-FOR 5’-GCATGGATCCATGGACCTATTGTTCGGGCG and the common GFP reverse primer 5’-GCATGCGGCCGCTTACTTGTACAGCTCGTCCA.

GFP-VPS4A WT and E228Q were inserted (BamHI/NotI) using the following primers:

FOR 5’- GCATGGATCCCCGCCACCATGGTGAGCAAG and REV 5’- GCATGCGGCCGCACTCTCTTGCCCAAAGTCC. Scrambled shRNA was a gift from David Sabatini (Addgene plasmid #1864) [343]. The shRNA construct used for CD63 knockdown (pLKO.1 TRCN0000007851) was obtained from the TRC 1.0 shRNA library at Penn State College of Medicine [344].

Lentivirus was produced in 293TN cells using X-tremeGENE HP DNA transfection reagent (Roche) and harvested according to the manufacturer’s protocol. For lentiviral transduction, subconfluent HDFs were transduced with lentivirus encoding tet-inducible constructs in the presence of 8 µg/mL polybrene (Sigma Aldrich). Transduced
cells were then passaged under 2 µg/mL puromycin (Thermo Fisher Scientific) selection. Following selection, cells were either used for infections with viruses described in a previous section, or seeded at subconfluence for a second lentiviral transduction with the EF1a-Tet3G lentivirus. Following the second lentivirus transduction with the EF1a-Tet3G lentivirus, cells were expanded and used for high MOI HCMV and HSV infections.

**Western blotting.** To harvest total cell lysates expressing dominant-negative ESCRT proteins, lysates were prepared from HCMV-infected cells at an MOI of 3 at 96 hours post infection by harvesting lysates in 1X Laemml Sample Buffer (BioRad) with 1 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin, 0.2 mM Na3VO4, 1 µg/ml leupeptin. Laemml Buffer was added directly to cell monolayers and incubated for 10 minutes before being collected and stored at -80°C. Lysates that did not contain dominant-negative ESCRT proteins were harvested at the indicated timepoints in RIPA buffer supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin, 0.2 mM Na3VO4, and 1 µg/ml leupeptin. For western blot analysis of EVs, aliquots were mixed 1:1 with urea lysis buffer containing 120 mM Tris-HCl pH 6.8, 5% SDS, 8 M Urea, 10mM EDTA, and 20% glycerol. Samples were analyzed by SDS-PAGE gel electrophoresis and immunoblotting on PVDF or nitrocellulose membrane blocked with 5% milk or 5% BSA in tris-buffered saline (0.1% tween). Horseradish peroxidase-conjugated secondary antibodies for western blotting detection were purchased from Santa Cruz (goat anti rabbit) and GE Healthcare (sheep anti mouse). Antibody dilutions were in accordance with manufacturer’s instructions. SuperSignal West Pico
Chemiluminescent Substrate (Thermo Scientific) was used for secondary antibody detection.

**Cell viability assays.** Cells used for viability assays were infected at an MOI of 3 and analyzed 96 hpi. 100 ng/mL doxycycline was added to samples at the indicated time points. For trypan blue experiments, cells were washed with PBS and treated with 0.05% Trypsin-EDTA (LifeTechnologies) and resuspended with media. Cells were mixed in 1:1 ratio with 0.4% Trypan Blue Dye (BioRad) and percentage live cells was measured using the TC20 Automated Cell Counter (BioRad). For the XTT assay, media was removed from all wells and replaced with fresh media at 96hpi. XTT and activating reagents (Biotium) were added to each well according to manufacturer’s instructions. Cells were incubated for 2 hours at 37°C and absorbance was measured using a Synergy H1 Hybrid Reader (BioTek). For CellTiter-Glo Luminescent Cell Viability Assay (Promega) the media was removed from all wells and replaced with fresh media at 96hpi. Reagents were added to each well according to manufacturer’s instructions. Luminescence was measured using the Synergy H1 Hybrid Reader.

**Epidermal growth factor degradation assay.** Fibroblasts were grown on coverslips in the presence or absence of doxycycline. Cells were cooled on ice for 10 min and washed 2 times with cold EGF binding media: DMEM 0.2% BSA 10mM HEPES pH 7.4. Cells were incubated on ice for 30 min in EGF binding media containing 100 ng/mL EGF conjugated to Alexa Fluor 555 (LifeTechnologies). Cells were washed 3 times with EGF binding media and returned to 37°C in EGF binding media. Cells were fixed at 30 and
180 min post 37°C incubation and coverslips were mounted using DAPI Fluoromount-G (SouthernBiotech). Images were taken on a C2+ Confocal Microscope System (Nikon).

**Immunofluorescence microscopy and imaging.** Coverslips containing either uninfected or HCMV-infected cells were fixed in 4% paraformaldehyde for 15 min at room temperature. Cells were blocked in PBS containing 10% human serum, 0.5% Tween-20, and 5% glycine. 0.1% Triton-X 100 was added for permeabilization. Primary and secondary antibodies were diluted in blocking buffer. FITC and Rhodamine-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. Alexa Fluor 488 and 568 secondary antibodies (Invitrogen) were also used. Coverslips were mounted with ProLong Diamond Antifade Mountant with DAPI (ThermoScientific). Images were taken on a C2+ Confocal Microscope System (Nikon). Images were processed using NIS elements software. Images shown are volume renderings of Z-stacks.

**Electron microscopy.** Cells were seeded on 60 mm Permanox tissue culture dishes (Nalge Nunc International). Cells were infected at an MOI of 3 for 96 hours and 100 ng/mL doxycycline was added 48hpi. At 96hpi cells were washed with PBS and fixed at 4°C in fixation buffer (0.5% [vol/vol] glutaraldehyde, 0.04% [wt/vol] paraformaldehyde, 0.1 M sodium cacodylate). Cells were processed by the Microscopy Imaging Facility (Pennsylvania State University College of Medicine). Briefly, the fixed samples were washed three times with 0.1 M sodium cacodylate, followed by postfixation in 1% osmium–1.5% potassium ferrocyanide overnight at 4°C. Samples were then washed 3 times in 0.1 M sodium cacodylate, dehydrated with ethanol, and
embedded in Epon 812 for staining and sectioning. For quantification of EM micrographs, tegumented capsids in contact with membrane were categorized as to whether envelopment was completed (enveloped) or in process (membrane-associated). For analysis of EVs, Equal numbers of uninfected or HCMV-infected EVs were transferred to 1 nm carbon film with a 10 nm formvar coated grid (Electron Microscopy Sciences) and briefly incubated with 2% uranyl acetate (Electron Microscopy Sciences). Images were acquired using a JEOL JEM-1400 Digital Capture transmission electron microscope.

**Quantitative real-time PCR analysis.** RNA was isolated from HDFs infected at an MOI of 3 at indicated timepoints using Qiagen RNeasy Mini kit, and cDNA was generated using Invitrogen SuperScript First Strand RT-PCR kit with oligo dT primers according to manufacturer’s instructions. qPCR was performed using Fast Start Universal SYBR Green Master (Roche) and StepOnePlus cycler (Applied Biosystems). Samples were normalized to GAPDH and fold change was calculated as compared to uninfected samples. Primers used were: CHMP6-FOR 5’-TTGGAAATGAGTGTCTGAAACAAG, CHMP6-REV 5’-CTGCGTCTCGTCCAGGAT; CHMP4B-FOR 5’-TCCCTCTATAGCCCTACCATCA, CHMP4B-REV 5’-TTCTCCAATTTCCTCATGTGC; CHMP2B-FOR 5’-AAAGTGATGAATTCCCAAATGAA, CHMP2B-REV 5’-TCTTCCACGAGACGCTTGAC, CD63-FOR 5’-ACCACACTGCTTCGATCCTG, CD63-REV 5’-TCTCCACACAGCCCTCCTTA; CD81-FOR 5’-CCTTCCACGAGACGCTTGAC, CD81-REV 5’-CGATCTTCTGGTGCGAGTCC;
GAPDH-FOR 5’ACCCACTCCTCCACCTTTGAC-3’, GAPDH-REV 5’-CTGTTGCTGTAGCCAAATTCGT.

**GW4869 experiments.** HDFs were seeded at subconfluence (30% confluency) and treated for 48 hours with 8 μM GW4869 (Cayman Chemical) or DMSO (Fisher) prior to infection. GW4869 or DMSO was not present during adsorption, but was added back at 3 hpi. Treatment groups were spiked with additional 2.5 μM GW4869 or DMSO at 2 dpi for high MOI experiments and at 2, 4, and 6 dpi for low MOI experiments. For EV rescue experiments, approximately 3,000-5,000 EVs per cell were added at 3, 4, and 5dpi.

**Extracellular vesicle isolation.** For EV isolation, cells were cultured in DMEM supplemented with 10% FBS that was centrifuged at 130,000 xg 4°C for 16 hours and subsequently filtered through a 0.22 μm filter to deplete the EVs. HDFs were seeded into eight T175 culture flasks and allowed to grow to confluency for 72 hours. This media was then collected and used to isolate uninfected EVs. Confluent flasks (approximately 8.0 x 10⁷ total HDFs) were then infected at an approximate MOI of 1 and infected cells were incubated for 72 hours before collecting the media. Media was collected and filtered through a 0.45 μm filter, and supernatant was subsequently centrifuged at 300 xg 4°C for 10 minutes, 1200 xg 4°C for 10 minutes, and 10,000 xg 4°C for 20 minutes. The pellet from the 10,000 xg step was resuspended in PBS and saved as the 10K pellet. Supernatant was transferred to ultracentrifuge tubes and centrifuged at 130,000 xg 4°C for 2 hours in an SW32Ti rotor. Supernatant was discarded and the pellet resuspended in 1 mL of 5% OptiPrep (Sigma-Aldrich). The resuspended pellet was loaded on top of a
stepwise OptiPrep gradient with 10% (1 mL), 15% (1 mL), 20% (1 mL), 30% (500 μL), and 41% (500 μL) steps and centrifuged in an SW55Ti rotor at 130,000 xg 4°C for 16 hours with no brake. Fractions were collected and brought to a final volume of 5 mLs in DPBS. Diluted fractions were centrifuged in an SW55Ti rotor at 130,000 xg 4°C for 2 hours. Pellets were resuspended in DPBS and stored at -80°C.

**Nanoparticle tracking analysis.** For each EV harvest, fractions positive for EV markers (CD63 and CD81) and negative for infectious virus were combined in equal volumes prior to NTA analysis. Samples were diluted to 1 mL with particle-free water. Each sample was loaded by syringe pump into the NanoSight NS300 (Malvern Instruments Ltd, Malvern, Worcestershire, UK) set in scatter mode, and five 60-second videos were captured. The flow cell was rinsed by 1 mL particle-free water 3 times between samples. Capture settings used were: screen gain of 1 and camera level of 14. Process settings used were: screen gain of 10 and detection threshold of 5. The size distribution and concentration of particles were calculated and exported using software NTA3.2 (Malvern Instruments Ltd). To generate a normalized size distribution between infected and uninfected EVs, the sum of vesicles within the defined diameter range were divided by the total number of vesicles with a diameter of 0-300 to get a relative proportion for that range. These proportions were then plotted with a Y axis from 0 to 1.

**Antibodies.** We used the following antibodies: CMV pp28 (5C3, Virusys), CMV pp52 (CH13, Santa Cruz), CMV pp150 (gift from David Spector), CMV gB (2F12; Virusys), CMV pp65 (7B4; a gift from David Spector [345], CMV pp71 (2H10-9; a gift from John
Purdy [346], CMV UL71 (1G; monoclonal antibody generated by Neil Christensen [306], CMV IE1/2 (a gift from Jim Alwine [341], Actin (EMD Millipore), EphA2 (Cell Signaling), CHMP6 (Proteintech), CHMP4B (Proteintech), CHMP4C (Origene), CHMP2A (Proteintech), CHMP2B (Proteintech), CHMP3 (Abcam), GFP (gift from John Wills), CD63 (TS63; Arigo Biolaboratories or H5C6; Developmental Studies Hybridoma Bank), CD81 (B-11; Santa Cruz Biotechnology), α-Tubulin (DM1A; Sigma-Aldrich), and Tsg101 (Fisher Scientific). Horseradish peroxidase-conjugated secondary antibodies for western blotting detection were purchased from Santa Cruz (goat anti rabbit) and GE Healthcare (sheep anti mouse). Antibody dilutions were in accordance with manufacturer’s instructions. SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) was used for secondary antibody detection.

Mouse monoclonal antibodies to HCMV UL71 (1G) and IE 1/2 (D4) were generated by Neil Christensen using similar methods that have been previously described [347, 348]. Full length UL71 and IE86 were fused to GST and purified from E. coli using glutathione sepharose beads (VWR). Purified proteins were injected into mice, and lymphocytes from the spleen of the mice were harvested. Lymphocytes were fused with mouse myeloma cells using polyethylene glycol. Supernatant was used to test antibody clones by ELISA using the original purified protein as antigen. Cells were clonally expanded and further tested by western blot and immunofluorescence to identify optimal clones for these applications.
Appendix A

Investigation of CHMP6 Recruitment to the HCMV cVAC
Out of all of the ESCRT-III subunits tested, CHMP6 was the only protein that accumulated in the cVAC during HCMV infection. The cVAC is made up of an accumulation of membrane from various vesicles and organelles. Given that the other ESCRT-III subunits were not recruited to the cVAC, we hypothesized that CHMP6 myristoylation is responsible for accumulation of the protein in this area of the cell. To test this, we generated a recombinant CHMP6 that was N-terminally fused to GFP, which should prevent myristoylation of the protein. We monitored localization of GFP-CHMP6 during HCMV infection. In support of our hypothesis, CHMP6 was dispersed throughout the cell and no longer accumulated at the cVAC (Figure A-1).

CHMP6 was inserted into pEGFPC1 by restriction digest with XhoI and EcoRI. The following primers were used: CHMP6-FOR-5’-

GCATCTCGAGCCATGGGTAACCTGTTCGGCCG, CHMP6-REV-5’-

GCATGAATTCCGAAGCTGCCACCAGCTCCG. GFP-CHMP6 was then inserted into pCDH by restriction enzyme digest using NheI and EcoRI. Plasmids were verified by Sanger sequencing, and pCDH GFP-CHMP6 was used to generate lentivirus as described in Chapter 5. Subconfluent HDFs were transduced with lentivirus 24 hours prior to HCMV infection. Cells were fixed at 96 hours post HCMV infection and immunofluorescence staining for the viral protein pp28 was used as a marker of the cVAC.
Figure A-1: CHMP6 localization during HCMV infection. GFP-CHMP6 expression in HCMV-infected (MOI=3) fibroblasts at 96 hpi. Immunofluorescence staining for the viral protein pp28 as a cVAC marker. Nuclei are stained with DAPI.
Appendix B

hnRNPA2B1 and SYNCRIPT Regulation During HCMV Infection
We analyzed protein and transcript expression levels of heterogeneous nuclear ribonuclear protein A2B1 (hnRNPA2B1) and synaptotagmin binding cytoplasmic RNA interaction protein (SYNCRIP) during HCMV infection (Figure B-1A). Protein and transcript levels of hnRNPA2B1 and SYNCRIP were both increased as infection progressed. Interestingly, sequence analysis of HCMV miRNAs identified the consensus binding sequences recognized by hnRNPA2B1 and SYNCRIP (Figure B-1B). Given the known targets of these miRNAs, the transfer of the miRNAs to uninfected cells may also contribute to the establishment of an environment more conducive to HCMV infection and enhance virus spread. However, the RNA binding proteins hnRNPA2B1 and SYNCRIP require cytoplasmic localization to incorporate miRNAs into EVs. Since these proteins are have primarily nuclear localization, we tested whether hnRNPA2B1 and SYNCRIP were present in the cytoplasm during HCMV infection. We analyzed nuclear and cytoplasmic levels of both RNA binding proteins (Figure B-1C). In uninfected cells, hnRNPA2B1 and SYNCRIP are undetectable in the cytoplasm, but both proteins accumulate in the cytoplasm as infection progressed to the later stages. Given the role of these proteins on miRNA packaging, cytoplasmic accumulation may affect the miRNAs loaded into EVs.

Total cell lysates were harvested from HDFs in RIPA buffer containing with 1 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin, 0.2 mM Na3VO4, and 1 µg/ml leupeptin. The following antibodies were used: hnRNPA2B1 (Proteintech), SYNCRIP/hnRNQ (IE84; Santa Cruz Biotechnology), and Tubulin (DM1A; Sigma-Aldrich). For qPCR analysis, RNA was isolated (Qiagen RNeasy Mini kit) from HDFs infected at an MOI of 3, and cDNA was generated using Invitrogen SuperScript First
Strand RT-PCR kit with oligo dT primers according to manufacturer’s instructions. qPCR was performed using Fast Start Universal SYBR Green Master (Roche) and StepOnePlus cycler (Applied Biosystems). Samples were normalized to GAPDH and fold change was calculated as compared to uninfected samples. The following primers were used: hnRNPA2B1-FOR 5’-GTGGAGCCAAAAACGTGCTGT, hnRNPA2B1-REV 5’-GCCAAAGCCTTTTTTCCTTCCA; SYNCRIP-FOR 5’- AAAGAAGCAGCTCAGGAGGC, SYNCRIP-REV 5’-GACGTCTGTAAGACCCTCTGT; GAPDH-FOR 5’ACCCACTCCTCCACCTTTTGAC-3’, GAPDH-REV 5’-CTGTTGCTGTAGCCAAATTGT.

To generate nuclear and cytoplasmic lysates, cells were scraped into cold DPBS, transferred to 1.5 mL Eppendorf tubes, and centrifuged at 2,000 rpm 4°C for 10 minutes. Supernatant was discarded and cells were resuspended in NAR A buffer (10mM HEPES pH7.9, 10mM KCl, and 0.1mM EDTA) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin, 0.2 mM Na3VO4, and 1 µg/ml leupeptin). After cell resuspension NP40 was added to a final concentration of 0.2% and vortex for 20 seconds. The suspension was centrifuged at 4,000 rpm 4°C for 2 minutes. After this step, the pellet contains intact nuclei, and the supernatant is the cytoplasmic fraction. Supernatant (cytoplasmic fraction) was transferred to a new tube and centrifuged at 14,000 rpm 4°C for 90 minutes. Following this step, supernatant was stored as cytoplasmic fraction at -80°C. the nuclei were resuspended in NAR A buffer (no inhibitors) and centrifuged at 3,000 rpm 4°C for 2 minutes. Supernatant was discarded and pellet was resuspended in NAR C buffer (20mM HEPES pH 7.9, 0.4 M NaCl, and 1mM EDTA) and vortexed at 4°C for 60 minutes. Suspension was centrifuged at 14,000
rpm at room temperature for 20 minutes. Supernatant was transferred to new tube at
stored as nuclear fraction at -80°C.
Figure B-1: Cytoplasmic accumulation of hnRNPA2B1 and SYNCRIP during HCMV infection. A) Western blot and qPCR analysis of hnRNPA2B1 and SYNCRIP expression in uninfected or HCMV-infected (MOI=3) fibroblasts at 24, 48, 72, 96 hpi. qPCR data represents three independent experiments. For western blot analysis, tubulin was used as a loading control. B) List of HCMV miRNA sequences with potential binding sequences recognized by either hnRNPA2B1 or SYNCRIP. Binding sequences are shown in red. Function of each miRNA and corresponding references are shown in green. C) Cytoplasmic fractions of uninfected and HCMV-infected (MOI=3) fibroblasts. Tubulin was used as a cytoplasmic loading control. Nuclear lysate (Nuc Lys) harvested from fibroblasts.
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