THE ROLE OF pUL138 IN HCMV PERSISTENCE

by

Alex S. Petrucelli

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As members of the Dissertation Committee, we certify that we have read the dissertation prepared by Alex S. Petrucelli entitled: The Role of pUL138 In HCMV Persistence. We recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

_____________________________ Date: 4/14/11
Dr. Felicia Goodrum

_____________________________ Date: 4/14/11
Dr. Nafees Ahmad

_____________________________ Date: 4/14/11
Dr. Lonnie Lybarger

_____________________________ Date: 4/14/11
Dr. Maggie So

Final approval and acceptance of this dissertation is contingent upon the candidate’s submission of the final copies of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

________________________________ Date: 4/14/11
Dissertation Director: Dr. Felicia Goodrum
STATEMENT BY AUTHOR

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SIGNED: Alex S. Petrucelli
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DEDICATION

To my loving family…if you should ever dare to read this!
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ABSTRACT

Human cytomegalovirus (HCMV) coexists indefinitely in infected individuals through a poorly characterized latent infection in hematopoietic cells. We previously demonstrated a requirement for UL138 in promoting a latent infection in CD34+ hematopoietic progenitor cells (HPCs). UL138 is encoded on three co-terminal transcripts of, 1.7-, 2.7-, and 3.6-kilobases. Interestingly, the UL138 protein product (pUL138) is necessary but insufficient for HCMV latency. The mechanisms by which pUL138 contributes to the latent infection are unknown, however other viral determinants are required for the latent infection. We identified 3 novel proteins pUL133, pUL135, and pUL136 encoded on the UL138 transcripts. Similar to pUL138, pUL133, pUL135, and pUL136 are Golgi localized type I transmembrane proteins expressed with early kinetics during productive infection. We have named these UL138 related proteins, CLAMPs for HCMV Latency Associated Membrane Proteins. Through a systematic immunoprecipitation analysis, we identified interactions between the CLAMPs and characterized an interaction between pUL133 and pUL138. Further, we mapped the interacting region to a specific domain in the C-terminal, cytosolic tail of pUL138. Additionally, we show that each of the CLAMPs has the ability to self-associate. The localization of the CLAMPs to the Golgi suggests that these proteins likely promote HCMV latency through a novel mechanism involving Golgi functions. Additionally, through a Y2H screen of a human bone marrow cDNA library, we identified an interaction between pUL138 and the heat shock
protein 40 (Hsp40) variant MRJ. We confirmed this interaction in mammalian cells and mapped the pUL138 region responsible for this interaction to a domain in the cytoplasmic tail of pUL138. We also demonstrated additional MRJ interactions with pUL133 and pUL136. Importantly, pUL138 specifically interacts with Hsp40 variants during productive infection. Preliminary data suggest that HCMV infection up regulates MRJ mRNA expression and recombinant viruses lacking pUL138 show a disproportionate up regulation of MRJ. pUL138 is the first HCMV protein demonstrated to promote a latent infection. While the mechanisms by which pUL138 contributes to latency remain unknown, the interaction with other CLAMPs and with MRJ, suggest that pUL138 may cooperate with other CLAMPs to modulate the cellular stress response at the Golgi to promote HCMV latency.
CHAPTER 1: LITERATURE REVIEW

Herpesvirideae

The Herpesvirideae are a family of large double stranded DNA viruses, which typically cause disease in animals. Within the herpesvirus family there are currently eight identified endemic human herpesviruses (human herpesvirus 1-8, HHV1-8) (83) and one zoonotic herpesvirus, McHV1, better known as Monkey B Virus (46). The hallmark of all herpesviruses is the ability to establish a persistent latent infection for the lifetime of their respective hosts. The herpesvirus family is divided into three subfamilies, the alphaherpesviruses, betaherpesviruses and gammaherpesviruses based on virion structure (40) and on other unique biological properties (125). The alphaherpesvirus replicate rapidly in culture, they exhibit a variable host range, and they establish latency primarily in sensory neurons (125). The human alphaherpesviruses include, Herpes simplex Virus Type-1, Herpes simplex virus Type-2, and Varicella Zoster virus (VZV) the virus that causes chicken pox (83). The betaherpesviruses are characterized by a narrow host range, a long replicative cycle, the ability to cause an enlarged cell-phenotype known as cytomegalia in infected cells (125) and they establish latency in cells of the myeloid lineage. Human herpesviurses 6 and 7 (HHV-6 and HHV-7) and human cytomegalovirus are the known human betaherpesviruses (83). The gammaherpesviruses exhibit a restricted host range and typically infect lymphoid cells including T and B cells where they establish latent infections (125). The two identified human gammaherpesviruses are
Epstein-Barr Virus (EBV), the virus responsible for infectious mononucleosis, and Kaposi’s sarcoma-associated herpesvirus (KSHV) the virus responsible for Kaposi’s sarcoma in AIDS patients.

All herpesviruses share several important biological properties. Morphologically the herpesviruses virions look similar in that they share a 4-part virion structure consisting of the viral DNA genome, nucleocapsid, tegument and lipid bilayer envelope (40, 83) (Fig. 1). The viral genome is a large (125-240 kbp) linear double stranded DNA genome (40, 83) capable of encoding hundreds of viral factors involved in all stages of the viral lifecycle. The genome is encapsidated by an icosahedral protein capsid that protects and contains the viral genome (Fig. 1). The nucleocapsid is in turn surrounded by a tegument layer rich in both viral and cellular factors (171) (Fig. 1) that have been shown to be involved in processes as diverse as initiating viral infection (8, 26, 82) to neutralizing the host cell’s innate immune response (1, 21, 22, 28, 107, 161, 162). The tegument is further enclosed in a lipid bilayer envelope studded with several viral glycoproteins required for virus attachment to, and entry into, the host cell (25, 63, 83, 146) (Fig. 1). Additionally, all herpesviruses replicate their genomes and assemble their capsids in the nucleus and inevitably kill the cell when viral replication is successfully completed (125). Most importantly though, through unique mechanisms, all herpesviruses persist in their respective hosts by way of a lifelong latent infection during which few viral factors are expressed and little to no progeny virus is produced (73, 140, 147).
Herpesviruses have a linear double stranded DNA genome that is protected by an icosahedral nucleocapsid. The nucleocapsid is surrounded by the tegument layer rich in viral and cellular proteins. The viral envelope is derived from Golgi membranes and is studded with viral glycoproteins. Virion components are labeled accordingly.

**Human Cytomegalovirus**

Human cytomegalovirus (HCMV) is an ancient and ubiquitous betaherpesvirus that persists in 60-90% of the world’s population through a lifelong latent infection (6, 43, 85). HCMV infection in the immunocompetent individual is typically asymptomatic but can occasionally cause a mononucleosis syndrome not unlike that caused by EBV (49, 139). Following primary infection, the virus establishes a persistent latent infection that is controlled primarily by T-
cell mediated mechanisms (38) but is never cleared. Most HCMV infections occur in childhood except in children from economically well developed countries where the overall HCMV seropositivity tends to be lower (17). Interestingly, HCMV seroprevalence is higher in populations of lower socioeconomic status (6, 43, 186) suggesting environmental factors play a role in HCMV transmission. Additionally, seroprevalence increases universally with age likely as a result of environmental and behavioral factors including child rearing practices, sexual activity, and overall living conditions (17). HCMV infection has also been associated with several risk factors such as age, female gender, and immune competence (43, 61). As a betaherpesvirus, HCMV exhibits a strict human tropism, has a long replicative cycle and has the ability to infect all the cells of the human body. Following infection, the virus can be detected in bodily fluids of infected individuals and can be transmitted through saliva, breast milk, and sexual contact (17, 60, 117, 124). Throughout the life of the host, periodic subclinical reactivation of the virus results in low levels of virus in the various bodily fluids allowing the virus to spread and persist in the human population.

Unlike infection in the immunocompetent population, HCMV infection in immunocompromised individuals is a significant cause of disease. HCMV is an opportunisitc pathogen in patients with immune deficiency including AIDS patients (104, 130, 151), patients undergoing intensive immunosuppressive therapy (12, 114), or stem cell transplant (SCT) (20, 29) and solid organ transplant (SOT) recipients (81, 126, 136). In AIDS patients, HCMV disease
typically manifests as retinitis that can lead to blindness (48, 151, 184) but with implementation of highly active antiretroviral therapy (HAART) the incidence of HCMV disease has decreased substantially (104, 130). HCMV disease in the stem cell transplant setting occurs either at early times post-transplant usually resulting in enterocolitis and pneumonitis (14, 15) or at later times resulting in similar pathologies as well as the potential for encephalitis and retinitis in rare cases (15). HCMV disease in solid organ transplant patients typically presents as fever, malaise, leukopenia, and macular rash but can also result in tissue-invasive diseases as diverse as pancreatitis, hepatitis, or myocarditis (67). Fortunately, in immunocompromised patients, the prophylactic use of antivirals like ganciclovir (GCV), which prevent viral replication, has aided in the prevention of HCMV disease (56). However, several factors including cost, toxicity, and overall drug effectiveness in different stages of HCMV disease, have proven problematic and have therefore hindered the use of GCV as a universal HCMV treatment (56).

Perhaps most notably, primary HCMV infection during pregnancy is the leading cause of infectious disease related birth defects in the developed world, affecting 1 in 100 live births in the United States (105). In particular, congenital HCMV infection is the leading infectious cause of deafness (47). While reactivation of latent virus in an HCMV seropositive mother can be problematic for the neonate, it is the primary infection in an HCMV seronegative mother that carries a 30-40% risk of congenital infection depending on the time of infection.
In particular, infection during the first trimester carries the highest risk of congenital infection and often results in the most severe manifestation of HCMV disease in the newborn (106). Importantly, many of the sensory neurological defects associated with congenital HCMV infection develop well after birth meaning that we have only just begun to understand the full impact of congenital HCMV infection on the human population.

Like all herpesviruses, HCMV has a linear, double stranded DNA genome. At 230-kilobases HCMV has the largest genome of all the human herpseviral genomes and its nearest relative, chimpanzee cytomegalovirus (CCMV) has the largest genome of all herpesviruses (41). The betaherpesvirus subfamily also exhibits the most genetic diversity of the various herpesvirus subfamilies (42) which may indicate an evolutionary mechanism to adapt to different hosts. The genome of HCMV is divided into unique long (UL) and unique short (US) regions each flanked by inverted repeat regions that are involved in packaging of the viral genome during viral replication as well as in mediating genomic isomerization (96). With approximately 200 predicted open reading frames (ORFs), many of which have yet to be characterized, the coding capacity of the HCMV genome is extensive.

Productive infection begins when an HCMV virion attaches to and enters a human host cell. The mechanisms governing attachment and entry however, remain unclear. The initial tethering of the HCMV virion to the host cell is thought to be mediated through cellular heparan sulfate proteoglycans (HSPGs) (37) as
this is a conserved attachment mechanism across the herpesvirus family (16). At least 10 viral glycoproteins have been identified in HCMV virions (171), 5 of which (gB, gH, gL, gM, and gN) have been shown to mediate attachment in various cell types (96) (19) (74). The most likely viral attachment and entry receptors are two glycoprotein complexes gH/gL/gO and gH/gL/pUL(128,130,131A) which have been shown to mediate cell specific entry and spread in various cell types (2, 51, 59, 128, 135, 175, 176). Depending on which cells the virus originates from, the progeny virions will contain different glycoprotein complexes which in turn allow for differential entry and spread in different cell types (135). Further, the difference in glycoprotein expression also determines whether HCMV enters the cell by membrane fusion or pH dependent endocytosis. Viruses expressing the gH/gL/gO complex enter fibroblasts by membrane fusion (36) whereas viruses expressing the gH/gL/pUL(128, 130, 131A) complex enter fibroblasts through pH-dependent endocytosis (134) suggesting that HCMV may utilize different entry pathways depending on both the cell type that the virus originated from and the cell type which the virus is currently infecting.

Upon entry, the viral capsids containing the viral genome and the surrounding tegument layer composed of various viral factors are deposited in the cell cytoplasm. Here the viral tegument proteins trigger the cellular innate immune response as well as initiate viral replication (discussed in herpesviridae section). Viral capsids are then delivered to the nucleus by an as yet unknown
mechanism that likely involves the microtubule network and nuclear pores (96). Once in the nucleus, viral genes are expressed in a temporally regulated cascade to begin viral genome replication and production of viral proteins needed for virus assembly and maturation. The HCMV genes are classified into three groups, immediate early (IE), early (E), and late (L), based on their expression kinetics during productive infection (96). Productive HCMV infection follows a highly orchestrated cascade of gene expression where the IE genes are expressed immediately upon infection, followed by the E genes which are required for viral replication, and the L genes, which mediate virion morphogenesis and maturation (96).

The process of virion morphogenesis and maturation are not well understood for HCMV. What is clear however, is that as the infection progresses, viral genomes acquire their capsids in the nucleus and are then enveloped with inner nuclear membrane as they exit the nucleus for assembly into virions (89). The acquisition of the tegument, the loss of the initial envelope (de-envelopment) and the acquisition of a second envelope (secondary envelopment) are still unclear though it appears that the final envelope is derived from the Golgi apparatus and the endoplasmic reticulum (89). Virions are finally released from the cell at the plasma membrane by exocytosis (96). Productive infection therefore culminates in the generation of infectious virions consisting of the viral genome enclosed by an icosahedral protein-rich capsid (T=16) surrounded by an icosahedrally ordered tegument layer (31) which is enclosed in a Golgi-
derived lipid membrane studded with viral glycoproteins (96). While HCMV is capable of infecting all cells of the human body, productive infection in vitro is restricted to primary fibroblasts, differentiated myeloid cells, endothelial cells, and some types of astrocyte cell lines (16) (68, 103). Productive HCMV infection and virus replication lead to cytopathic affects including cell swelling (cytomegalias), detachment and ultimately, cell death.

**HCMV Latency**

Productive infection is key to the production of progeny virus, however, the long-term persistence of HCMV is accomplished via a latent infection in its human host. Latency is a quiescent state of infection with little or no viral gene expression. During the latent infection, HCMV must maintain its genome, avoid the host immune system, and eventually reactivate to replicate productively (96). Latent HCMV infections are extremely prevalent, affecting 60-99% of the human population (96). Although typically asymptomatic, the chronic nature of latent HCMV infection has been linked to atherosclerosis (66, 115, 153, 157, 172, 173), age related immune senescence (115, 157, 172, 173) and chronic inflammation leading to frailty (18, 133, 170). Reactivation from latency is a significant cause of morbidity and mortality for immunocompromised individuals including HIV/AIDS patients, stem cell and solid organ transplant recipients and cancer patients receiving intensive immunosuppressive therapy (15, 45, 96). The antiviral ganciclovir, which targets cells containing replicating virus, is a common
treatment for active infection but it cannot target latent virus and, therefore, fails to clear latent virus. Latent HCMV, therefore, poses a significant health risk to an increasingly large proportion of the human population. A complete understanding of the viral and cellular mechanisms contributing to the latent infection will allow for the development of novel treatments to target latent virus and ultimately prevent HCMV disease.

While the cellular reservoirs and viral factors that promote the latent infections of EBV, HSV-1 and VZV have been well characterized; the viral and cellular mechanisms dictating the latent infection of HCMV remain unknown. HSV-1 and EBV establish latent infections in neurons and B-cells, respectively, but the latent reservoir for HCMV until recently remained controversial. Classically, HCMV has been studied in fibroblasts, which only support a productive infection, thereby prohibiting any understanding of the latent infection of HCMV. More recently however, models have been developed to study latent HCMV infection in cells of the myeloid lineage. In vitro experiments have demonstrated the presence of quiescent viral genomes in infected monocytes (142). Additionally, latent viral genomes have been detected in granulocyte-macrophage progenitor cells following infection (58, 77, 78). It is now thought that HCMV establishes latency in a primitive hematopoietic progenitor cell at an earlier stage of differentiation. Our laboratory has demonstrated that an infection phenotypically similar to the latent infection can be established in primitive hematopoietic progenitor cells (HPCs) (CD34+/CD38-) infected in vitro (52).
Figure 2 HCMV genome organization: Laboratory vs. Clinical Strains. HCMV genome organization: Laboratory vs. Clinical strains. Schematic of the organization of the HCMV genome (adapted from (99)). The HCMV genome is ~230 Kb and is divided into the unique long (UL) and unique short (US) regions flanked by inverted repeat regions. The general orientation of each segment is indicated by the associated arrow. Laboratory (high passage) strains have a duplication of the RL1-14 region while clinical (low passage) strains retain the ULb' region containing the predicted open reading frames (ORFs) UL133-UL152.

It is likely therefore, that cell-type specific differences contribute to the latent infection of HCMV. Along these lines, latent HCMV genomes have been detected in CD34+ HPCs from latently infected, healthy individuals (92, 174) suggesting that indeed the latent reservoir for HCMV is likely the primitive CD34+ HPCs of the bone marrow.

Considering the cell-type specific differences between fibroblasts and CD34+ HPCs, it is likely that infection of CD34+ HPCs follows a different course than productive HCMV infection of fibroblasts. Indeed, in vitro infection of CD34+
HPCs results in expression of a distinct subset of viral genes in the absence of viral replication (54). Overall, viral gene expression is reduced as viral latency is established in myeloid progenitor cells (33, 54). Additionally, virus can be reactivated from latently infected CD34+ HPCs and ultimately lead to productive infection of fibroblasts in vitro. Further, several HCMV factors are expressed in HPCs including antisense immediate early transcripts (78, 80), antisense UL81-82 transcripts (11), and a viral IL-10 homolog (72). The viral IL-10 homolog was also expressed in granulocyte-macrophage progenitor cells (GM-Ps) during infection with laboratory strains of HCMV that are incapable of establishing and maintaining a latent infection (discussed below) and failed to affect antigen presentation in neighboring cells (109), suggesting that although the viral IL-10 homolog is expressed in HPCs, its function may not be involved in viral latency. While these factors have been suggested to play a role in HCMV pathogenesis, none of these factors has been ascribed a functional role during the latent infection.

Cellular chromatin remodeling plays a critical role in the regulation of gene expression essential to various cellular processes including cell cycle regulation, apoptosis, and the response to microbial infection. Chromatin remodeling has also been implicated in viral latency and reactivation as chromatin modifications of the HCMV genome have been shown to modulate viral gene expression (101). Further, HCMV can be reactivated by cellular differentiation (121, 122) as well as by activation of various cellular signaling pathways involved in allogenic
stimulation and the cells reaction to pro-inflammatory cytokines (118, 144). The complete role of chromatin modifications of the viral genome in HCMV latency and reactivation is still poorly understood but promises to hold key insights into the mechanisms governing HCMV persistence.

Until recently, most HCMV research has utilized lab-adapted viral strains that have undergone serial passage in fibroblasts, rendering these strains nonpathogenic and fundamentally different from wild type clinical isolates of HCMV (Fig. 2). Extensive passage in fibroblasts resulted in various mutations, genomic rearrangements, and the loss of a ~15-kb region of the genome known as the ULb’ region (Fig. 2). Viruses lacking the ULb’ region are unable to establish or maintain a latent infection in primary CD34+ HPCs infected in vitro (53). Additionally, lab-adapted HCMV strains exhibit restricted cell tropism, increased growth kinetics, increased virus titers, and increased cytopathic effects. These phenotypic differences have therefore made clinical isolates the cornerstone of HCMV latency research with the primary focus on potential genes encoded within the ULb’ region (Fig. 3). The ULb’ region encodes ~20 putative ORFs, UL133-UL151 that are dispensable for viral replication in fibroblasts (30, 42, 98, 100) (Fig. 3). Most of the putative ORFs encoded within the ULb’ region however, remain uncharacterized.
Figure 3 HCMV ULb' region. Schematic of the organization of the ULb' region unique to clinical (low passage) strains of HCMV. The predicted ORFs UL133-UL152 are indicated in their relative positions within the ULb' region. The HCMV latency determinant UL138 is highlighted in red.

Since the ULb' region is unique to clinical HCMV stains and is dispensable for virus growth in culture, it has been proposed that the ULb' region likely encodes factors involved in viral replication, persistence and pathogenesis in the human host (30, 42, 100, 177). Not surprisingly then, the ULb' genes that have been identified and at least partially characterized, have been shown to be involved in viral persistence and dissemination. UL141 and UL142 have been shown to protect infected cells from NK lysis (167, 182). UL141 prevents NK activation by sequestering CD155 in the ER (167). CD155 is a receptor for NK cell activating receptors and its sequestration in the ER prevents maturation and surface expression, thereby inhibiting NK activation (167) and subsequent immune surveillance of infected cells. Additionally, UL142 encodes an MHC-1-
like molecule that protects fibroblasts from NK lysis when expressed exogenously (182) here again preventing immune clearance. The gene product of UL144 which belongs to the tumor necrosis factor α (TNFα) receptor superfamily activates NFκB thereby stimulating the cellular chemokine CCL22 and promoting TH2 immune responses and perhaps aiding viral spread and dissemination (113). UL146 and UL147 have sequence similarity with α (CXC) chemokines and act as potent chemoattractants for the recruitment of neutrophils to aid in viral dissemination (108). We previously identified ULb' sequences including putative ORFs UL136-UL142 that were required for latent HCMV infection of CD34+ HPCs infected in vitro (53). Recombinant viruses lacking UL138 failed to establish a latent infection in CD34+ HPCs infected in vitro (53). Additionally, UL138 transcripts were detected in latently infected, healthy, seropositive individuals (53). UL138 is therefore the first identified HCMV encoded determinant required for the latent infection (53).

The success of a latent HCMV infection requires that the virus must maintain its genome, avoid the host immune system, and eventually reactivate from latency to replicate productively (96). In order to accomplish these goals, the virus must tightly control the replication of its genome under the ever-changing cellular conditions so that reactivation from latency occurs at an optimal time for the virus to replicate, disseminate and spread within the human population. While genes encoded within the ULb' region have already been shown to be involved in host immune modulation it remains unknown whether
additional ULb' genes are involved in viral replication, persistence or reactivation. Interestingly, UL138 was shown to be required for HCMV to establish and maintain the latent infection in vitro (53). The mechanisms through which UL138 promotes HCMV latency are unknown. However, it is likely that UL138 promotes viral latency through modulation of key cellular pathways involved in cell-survival and differentiation as HCMV reactivation has been clearly associated with cellular differentiation (53, 68, 144). Importantly, cellular differentiation may not be sufficient for viral replication as cytokine- and TLR-mediated signaling have also been shown to induce reactivation (58, 143, 145) thereby implicating the innate immune response and perhaps cell-survival strategies in modulation of HCMV latency.
CHAPTER 2: CHARACTERIZATION OF A NOVEL GOLGI-LOCALIZED LATENCY DETERMINANT ENCODED BY HUMAN CYTOMEGALOVIRUS *

Introduction

Human cytomegalovirus (HCMV) is an ancient herpesvirus that persists in 60-99% of the human population worldwide through a latent infection that is asymptomatic in healthy individuals (96). Reactivation of HCMV from latency can result in life-threatening pathology in immunocompromised individuals including stem cell and solid organ transplant recipients, AIDS patients, and cancer patients undergoing intensive chemotherapy (15, 45, 96). In addition to overt pathologies associated with reactivation from latency, the impact of viral coexistence on our biology is ill-defined but encompasses a wide range of possibilities including conferring protection from other microbial infections (9), contributing to the development of vascular disease (66, 153), or gradually exhausting the host’s immune defenses (115, 157, 172, 173). Viral persistence or latency is a poorly understood phenomenon in virology, yet it is critical to understanding how viruses assimilate into and impact our biology and cause disease.

HCMV latency is best characterized in hematopoietic cells of the myeloid lineage. While the primary cellular reservoir for latent HCMV is unknown, latency has been studied experimentally in a wide variety of primary progenitor cells including CD34+ and CD34+/CD38- cells (52-54, 87, 187) and myeloid-lineage

cells including granulocyte-macrophage progenitor cells (GM-Ps) (58, 77, 78) and CD34\(^+\)-derived dendritic cells (121). Indeed, latent HCMV genomes have been detected in CD34\(^+\) cells (92, 174), GM-Ps (58, 78, 141), and monocytes (94, 144, 163, 164) from latently infected, healthy individuals. HCMV latency and reactivation from latency are intimately associated with hematopoietic cell differentiation (143-145, 164). Because HCMV genomes are detected in cells as primitive as CD34\(^+\) progenitor cells in the hierarchy of hematopoietic differentiation (92, 138, 174), we have chosen primary human CD34\(^+\) hematopoietic progenitor cells (HPCs) as the basis for our studies.

The contribution of viral determinants to latency has been poorly characterized. Several HCMV transcripts and proteins have been detected in hematopoietic cells infected in vitro or derived from healthy, seropositive individuals. First, transcripts originating from the major immediate-early (IE) region via alternative start sites and the proteins they encoded were detected following infection of GM-Ps in vitro and in latently infected individuals (78, 80). Second, a variant of the viral IL-10 homologue encoded by UL111.5A was detected in GM-Ps infected in vitro and in monocytes from seropositive individuals (72). Third, a transcript antisense to the UL81-82 genes was detected in monocytes from healthy seropositive individuals (11). Despite the expression of these factors in hematopoietic cells infected endogenously or in vitro, a role has yet to be demonstrated for the latency-associated IE transcripts or the UL81-82 and UL111.5A gene products in HCMV latency. Further, the ORF94 protein...
encoded by the latency-associated IE transcripts was dispensable for establishing and maintaining a latent infection in vitro (181). We previously identified sequences including the putative ULb’ genes, UL136-142, that are required for an infection with the hallmarks of latency in HPCs infected in vitro (53). Specifically, recombinant viruses lacking the UL138 ORF exhibited a loss of latency phenotype and replicated productively in HPCs infected in vitro. Further, UL138 transcripts were detected in CD34+ cells and monocytes from healthy seropositive individuals (53).

UL138 is a previously uncharacterized putative HCMV ORF. Our present study represents an initial characterization of the UL138 gene products and investigates their role in the latent infection. In productively infected fibroblasts, UL138 is encoded on two large, polyadenylated, co-terminal transcripts of 2.7- and 3.6-kb that are co-linear with the viral genome. These transcripts give rise to a 21-kDa protein (pUL138) corresponding to the predicted coding sequence of UL138. pUL138 was required for an infection with the hallmarks of latency in vitro. However, our data suggests that the UL138 transcripts or other ULb’ ORFs encoded between UL133 and UL142 also contribute to the in vitro latency phenotype. pUL138 localizes to the Golgi as an integral type-I transmembrane protein where the majority of protein resides on the cytoplasmic face of the Golgi membranes. Given the localization of pUL138 in the Golgi, pUL138 may contribute to latency by mediating protein trafficking or the cellular stress response. pUL138 is the first virus-coded protein demonstrated to function in
HCMV latency in HPCs infected \textit{in vitro}. Further characterization of pUL138 in HPCs and the mechanisms by which pUL138 functions in latency is the focus of our ongoing research.

\textbf{Materials and Methods}

\textbf{Cells}

Human lung fibroblasts (MRC5) (ATCC, Manassas, VA) were cultured in Dulbecco’s modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 100 U/mL penicillin, and 100 \(\mu\)g/ml streptomycin. Human astrocytoma cells (U373) (ATCC) were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 \(\mu\)g/ml streptomycin. 293 cells (ATCC) were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 \(\mu\)g/ml streptomycin. ARPE-19 cells (ATCC) were cultured in a 1:1 mixture of DMEM and Ham’s F-12 media supplemented with 1.2 g/L sodium bicarbonate, 2.5 mM L-glutamine, 15 mM Heps, 0.5 mM sodium pyruvate, and 10% FBS. The M2-10B4 murine stromal cell line expressing human interleukin-3 (IL-3) and granulocyte-colony stimulating factor (G-CSF) and the S1/S1 murine stromal cell line expressing human IL-3 and stem cell factor (SCF) were generously provided by Stem Cell Technologies Ltd. on behalf of D. Hogge (Terry Fox Laboratory, University of British Columbia, Vancouver, British
Columbia) and cultured as recommended (93). All cells were maintained at 37°C with 5% CO₂.

For most experiments, bone marrow cells were obtained from waste produced during bone marrow harvest from healthy donors at the University Medical Center at the University of Arizona via a protocol approved by the Institutional Review Board. Alternatively, fresh bone marrow from cadavers was purchased from the National Disease Research Interchange (Philadelphia, PA). CD34⁺ cells were isolated as described previously (54).

**Viruses**

The FIXwt virus strain has been cloned as a bacterial artificial chromosome (BAC) and engineered to express the green fluorescent protein (GFP) (unpublished results, D. Yu and T. Shenk). Virus stocks were propagated by electroporation of infectious BAC DNA into MRC5 cells and purified by density gradient centrifugation through a 20% D-sorbitol cushion at 20,000 RPM in an SW28 rotor (Beckman Coulter, Fullerton CA) for 80 min at 22°C. Virions were resuspended in IMDM containing 2% bovine serum albumin (BSA) and stored at -80°C. Infectious virus yields were determined by tissue culture infectious dose (TCID₅₀) on MRC5 fibroblasts.

Recombinant viruses were constructed in E. coli by linear recombination in a 2-step positive/negative selection that leaves no trace of the engineering process (178, 183). For FIX-UL138STOP, PCR fragments encoding galK with
homologous flanking sequences to the targeted region of the HCMV genome in the FIX\textit{wt} strain were synthesized using forward (5’-CCATGGACGATCTGCCGCTGAACGTCGGGTTACCCATCATCGGCCTGCTTGACAATTAATC-3’) and reverse (5’-TCGTGCCAATGGTAAGCTAGATAGCAGAGAATGGCCA CGATCAGCAGAGTCAGCAGCTGTCTGCTCTCTT-3’) primers and the pGalK plasmid (50 ng) (178). The primer sequences specific to \textit{galK} are underlined and the remaining portion of the primer is homologous to HCMV sequences flanking the targeted region. \textit{GalK} PCR products were digested with DpnI, gel purified, and electroporated into recombinogenic SW102 \textit{E. coli} containing the FIX\textit{wt}-BAC. \textit{GalK} positive recombinants were selected for growth on M63 minimal plates and screened on McConkey agar plates, both supplemented with 0.2% galactose and 15 µg/ml chloramphenicol. In the second step, 100-bp complementary oligos containing \textit{UL138} with a stop codon substituted for codon 15 (ATG→TAA) (5’-CCATGGACGATCTGCCGCTGAACGTCGGGTTACCCATCATCGGCCTGTAAC TCGTGCTGATCGGAGCTATTCTCTAGTCTATCTAGTACCATTGGCAGA-3’) were annealed and electroporated into recombinogenic SW102 \textit{E. coli} containing the FIX-\textit{galK}\textsuperscript{+} BAC. \textit{GalK} recombinants were selected on M63 minimal plates containing 0.2% 2-deoxygalactose and 15 µg/ml chloramphenicol. Recombinants were further screened by BAC digestion, PCR and sequencing. Infectious BAC DNA was isolated and electroporated into MRC5 cells to produce virus stocks.
The recombinant FIX-UL138\textsubscript{myc} virus was constructed similarly by amplifying \textit{galK} using forward (5'-TGTACAAAAAGAGAGAGACTGGGACGTAGATCCGGACAGAGGACCGGTCACC
CCTGTTGACAATTAATCATCGGC-3') and reverse (5'-GTCAAAACGACATTACCGCGATC
CGCTCCCCTCTTTTTTCTTTTTCTCATTCAGCAGCTGCTGCC-3') primers and the PCR product was substituted for \textit{UL138} in the FIX\textit{wt}-BAC genome in SW102 \textit{E. coli}. The primer sequences specific to \textit{galK} are underlined and other sequences are specific to sequences flanking \textit{UL138}. \textit{GalK} was then replaced with a PCR product encoding \textit{UL138} with an in-frame, C-terminal myc epitope tag. This PCR product was synthesized using the FIX\textit{wt}-BAC as template and the forward (5'-TCATCCGCGACTCTACGAC-3') and reverse (5'-GTCAAAACGACATTACCGCGATCCGCTCCCCTCTTTTTTCTTTTTCTCATtcA
AGATCCTCTTTCTGAGATGAGTTTTTGTTCGATGTATTCTTGATGATAAATGTAC
C-3') primers where the myc epitope sequences are underlined, the stop codon is lower case, and HCMV sequences are in uppercase. Recombinant clones were selected as described (178) and screened by restriction digest, PCR and sequencing.

The replication of recombinant viruses was determined by infecting MRC5 cells at an MOI of 0.01. Cells and media were collected over a 20 day time course and sonicated. Virus titers in each lystate were determined by TCID\textsubscript{50} on MRC5 cells.
Northern Blotting

Total RNA was isolated from cells infected at an MOI of 2 using TRIzol LS Reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Where indicated, polyadenylated RNA was further purified using the NucleoTrap mRNA Mini Kit (Macherey-Nagel, Bethlehem, PA). To analyze transcript stability, 4 μg/ml actinomycin D was added to infected cells at 16 hpi and RNA isolated over a time course. RNA was glyoxylated (7), subjected to 1% agarose gel electrophoresis, and transferred to a Nytran nylon membrane using a Turboblotter (Whatman, Keene, NH). RNA was crosslinked to the membrane using a UV Stratalinker (Stratagene, La Jolla, CA). Antisense riboprobes recognizing ULb’ coding sequences were generated from PCR products (see primers, Table 1) cloned into the pGEM-T Easy vector (Promega, Madison, WI). Vectors were linearized and transcribed in vitro to generate [α-32P]-CTP-radiolabeled probes using the SP6/T7 Riboprobe Combination System (Promega). Probes were hybridized to blots in 4X SSC (0.6 M NaCl, 0.06 M sodium citrate [pH 7.0]), 5X Denhardt’s (0.01% w/v Ficoll 400, 0.01% w/v polyvinylpyrrolidone, 0.01% w/v BSA), 1% SDS, 50% formamide, and 10 μg/ml denatured salmon sperm at 60°C overnight. Membranes were washed (7) and hybridization detected by exposure to autoradiographic film or by phosphorilluminescence.
Table 1 Primers for Northern Analysis Templates.

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<th>Sequence</th>
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<td>5’-ATG GGT TGC GAC GTG CAC GAT C-3’</td>
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<td>5’-TTA CGT TCC GGT CTG ATG CTG C-3’</td>
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<tr>
<td>UL135</td>
<td>FWD</td>
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<tr>
<td></td>
<td>REV</td>
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</tr>
<tr>
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<td>FWD</td>
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</tr>
<tr>
<td></td>
<td>REV</td>
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</tr>
<tr>
<td>UL138</td>
<td>FWD</td>
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<tr>
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<tr>
<td>UL138b</td>
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<td>5’-AAA AAG AGG GGA GCG GAT CG-3’</td>
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<tr>
<td></td>
<td>REV</td>
<td>5’-ATC ACC GCC ACC ATT ACC AC-3’</td>
</tr>
<tr>
<td>UL139</td>
<td>FWD</td>
<td>5’-ATG CTG TGG ATA TTA ATT TTA TTT GC-3’</td>
</tr>
<tr>
<td></td>
<td>REV</td>
<td>5’-TCA CCG AGG CGG AGG TGG AAA T-3’</td>
</tr>
</tbody>
</table>

Rapid Amplification of cDNA ends (RACE)

Total RNA was isolated from infected MRC5 fibroblasts at 18 hpi, digested with DNase, and purified using the Nucleospin RNA-II kit (Macherey-Nagel). The 5’ ends of the UL138 transcripts were mapped using the SMART RACE cDNA amplification kit IIa (Clonetech, Mountainview, CA). A poly(dT)_{25}VN primer was annealed to 1 μg of total RNA and reverse transcribed with 200 U Superscript II (Invitrogen) with 7% DMSO for 90 min at 42°C. The 5’ end was amplified with primers to the SMART-IIa oligonucleotide and a gene specific primer (gsp) to the UL138 ORF (5’-CAACAGCGGATCCAGCCAGCG GTAGCTCAAA-3’) using Advantage 2 polymerase (Clonetech) under the following conditions: 7% DMSO, 1 M Betaine, 400 nM primer, 94°C for 30 sec, then 30 cycles of 94.5°C for 30 sec, 59°C for 30 sec, 72°C for 4.5 min, followed by a final extension of 10 min at 72°C. The 3’ ends of the transcripts were mapped using the 3’ RACE system,
version 2.0 (Invitrogen). Total RNA (1 µg) was annealed with a 3' adapter primer and reverse transcribed using 200 U Superscript III for 60 min at 55°C, followed by a 15 minute enzyme inactivation at 75°C and a 30 minute digestion with 2 U of RNase H. The 3' end of the transcript was amplified with the gsp to the 5' end of the UL138 ORF (5'-ATGGACGATCTGCCGCTGAA-3') and the Adapter Primer Mixture using Advantage 2 polymerase and the following conditions: 94°C for 30 sec followed by 30 cycles of 94°C for 30 sec, 65°C for 30 sec, then 72°C for 3.5 min. All RACE products were gel-purified, cloned into the pGEM-T Easy vector, and sequenced.

**Plasmids**

Wild type UL138 or UL138 containing a premature stop codon (TAA) at codon 15 (ATG) were PCR amplified from BAC DNA using the UL138 forward primer (5'-GGGGAAATTCCAGAGGACGGTCACCATGGAC-3') and the UL138 reverse primer (5'-GCGCTCTAGAGCCGTGTATTCTTGATGATAATG-3'). Synthesized fragments were digested and cloned in frame into the EcoRI and XbaI sites of pEF1/Myc-His (Invitrogen) generating carboxy-terminal-myc-tagged-UL138 expression vectors, pEF1-UL138WT and pEF1-UL138STOP. pGL3-MIEP1400 was created by PCR amplifying the entire HCMV MIEP including the modulator and enhancer region (91) and cloning it into the KpnI and HindIII sites of the pGL3-Basic firefly luciferase plasmid (Promega). The SV40 driven renilla luciferase plasmid pRL-SV40 was a generous gift of T. Bowden (University of
Arizona). The pSVH-1 IE1/2 expression plasmid was a generous gift from J. Nelson (Oregon Health Sciences University) (152).

**Immunoblotting**

Protein lysates (5-10 µg of protein per lane) were separated by SDS-PAGE and transferred to 0.22 µm nitrocellulose (Hybond-ECL, Amersham Biosciences, Piscataway, NJ) or 0.45 µm PVDF membranes (Immobilon-FL, Millipore, Billerica, MA). Membranes were blocked in Tris-buffered saline (TBS; 25 mM Tris pH 8.0, 137 mM NaCl, 3 mM KCl, 1.5 mM MgCl₂, pH 8.0) plus 5% nonfat dry milk and 2.5 mg/ml BSA overnight at 4°C. Blots were incubated in primary antibody in TBS supplemented with 2.5 µg/ml BSA and 0.05% Tween 20 (TBS-BT) for 1 h at room temperature. Primary antibodies are listed in Table 2. Blots were washed 3 times in TBS-BT for 15 min each, then incubated with either goat anti-mouse or goat anti-rabbit IgG secondary antibodies conjugated to horseradish peroxidase (Invitrogen) in TBS-BT plus 5% normal goat serum. Membranes were developed with ECL Plus Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ) according to manufacturer’s instructions and exposed to autoradiography film. Alternatively, protein blots were visualized using goat anti-mouse IgG (H+L), DyLight 680 Conjugated and goat anti-rabbit IgG (H+L), DyLight 800 conjugated secondary antibodies (Pierce, Rockford, IL) in conjunction with the Odyssey Infrared Imaging System (LI-COR, Lincoln, NE).
Latency and Reactivation

Freshly isolated CD34-enriched bone marrow cells were infected at a multiplicity of infection (MOI) of 2 PFU per cell for 20 h in Iscove’s modified DMEM (IMDM) supplemented with 10% BIT9500 serum substitute (Stem Cell Technologies, Vancouver, BC), 2 mM L-glutamine, 20 ng/ml low density lipoprotein (Sigma, St. Louis, MO), and 50 µM 2-mercaptoethanol. Following infection, cells were washed in citrate buffer (40 mM Na citrate, 10 mM KCl, 135 mM NaCl, pH 3.0) for 1 min to inactivate unabsorbed virus. Infected cells were labeled with CD34+ specific monoclonal antibodies conjugated to phycoerythrin (BD Biosciences Immunocytometry Systems, San Jose, CA). Pure populations of infected (GFP+) CD34+ HPCs were isolated by fluorescent-activated cell sorting (FACSAria, BD Biosciences Immunocytometry Systems) and cultured in long-term bone marrow cultures on irradiated (2000 Rads, Co60 γ-iradiator, Atomic Energy of Canada, Mississauga, ON; University of Arizona, Tucson, AZ) stromal cell monolayers as described previously (53, 93). Briefly, irradiated recombinant M2-10B4 murine fibroblasts engineered to express recombinant human IL-3 and granulocyte colony stimulating factor (G-CSF) and S1/S1 murine fibroblasts engineered to express recombinant human stem cell factor and IL-3 (a gift from DR. D. Hogge, Terry Fox Laboratory, BC provided through StemCell Technologies) are mixed at a 1:1 ratio and seeded onto collagen coated dishes at 1.5X10^5 cells per ml in human long-term culture media (hLTCM, Alpha Modification of Eagle’s Minimum Essential Medium supplemented with 12.5%
horse serum, 12.5% fetal bovine serum, 0.2 mM i-inositol, 20 µM folic acid, 0.1 mM 2-mercaptoethanol, 2 mM l-glutamine, and 1 µM hydrocortisone) (93). Infected CD34^+ cells are cultured above irradiated feeder monolayers in collagen-coated transwells (Costar, Corning, NY). To measure latency and reactivation at 11-14 days postinfection, 10,000 infected HPCs were in to each of 12-24 wells of a 96-well tissue culture plate containing MRC5 fibroblasts. To differentiate virus made as a result of reactivation from virus pre-existing in long-term cultures, an equal number of infected HPCs was mechanically disrupted and plated on fibroblasts. The fraction of wells containing GFP expressing fibroblasts was scored 20 days later (53).

**Luciferase**

U373 cells were seeded onto 24 well plates at 1x10^5 cells per well 2 days prior to transfection. Cells were transfected using Lipofectamine 2000 (Invitrogen) in Opti-MEM I (Invitrogen). Briefly, U373 cells were incubated with 4 µg of Lipofectamine 2000 and 10 ng pGL3-MIEP_1400 firefly reporter plasmid, 20 ng pRL-SV40 renilla reporter plasmid and 0.1, 0.5, 1.0, or 3.0 µg pEF1-UL138_{WT} or pEF1-UL138_{STOP} effector plasmid compensated with pEF1-EMPTY effector plasmid to maintain 3 µg total effector DNA per transfection. At 6 h post-transfection, media was replaced with normal growth media. Luciferase activity was assayed 48 h post-transfection using the Dual-Luciferase Reporter Assay System (Promega) and a Berthold FB12 luminometer (Berthold Detection
Systems, Oak Ridge, TN) according to manufacturers’ instructions. Relative luciferase activity was calculated by normalizing the firefly luciferase activity to the renilla luciferase activity.

293 cells were seeded onto 12 well plates at $1 \times 10^5$ cells per well and transfected with 1.5 µg Lipofectamine 2000 per well. 293 transfections included 10 ng pGL3-MIEP$_{1400}$ firefly reporter plasmid, 20 ng pRL-SV40 renilla reporter plasmid, 100 ng pSVH-1 IE1/2 expression plasmid, and 0.1, 0.5, 1.0, or 1.5 µg pEF1-UL138$_{WT}$ or pEF1-UL138$_{STOP}$ effector plasmid compensated with pEF1-EMPTY effector plasmid to maintain 1.5 µg total effector DNA per transfection. Transfections were performed and assayed as discussed above.

**Real-time reverse transcriptase PCR**

RNA was isolated and DNase-digested using the NucleoSpin RNA II kit (Macherey-Nagel) according to manufacturer’s instructions. cDNA was generated using oligo d(T) primers and Superscript II Reverse Transcriptase (Invitrogen) according to manufacturers instructions. Quantitative real-time PCR was performed using D-Lux primers (Invitrogen) specific for UL122 (5’-cgggACAGGAAGACATCAAGCCcG-3’ and 5’-TTGTTGCGGTACTGGATGGTAAA-3’) and UL138 (5’-cgggCGTCGATCTGTTGAAACCcG-3’ and 5’-CATGGCTACGTTGTTGAAACTG-3’). Linear fragments for the generation of UL122 and UL138 standard curves were synthesized using primer sets specific for the targeted HCMV genomic
regions: for UL122 exon 5 (5'-GACAACCCACTCTTGAGCG-3' and 5'-ATTGCGCACCTTCTCAGTG-3') and for UL138 (5'-ATGGACGATCTCCCCTGAA-3' and 5'-TCACGTGTATTTCTGATGATAA-3'). Real-time PCR reactions were run on an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) using Platinum PCR SuperMix-UDG with ROX (Invitrogen). Reaction conditions were as follows: 50°C for 2 min, 95°C for 10 min, 60 cycles of 95°C for 15 sec then 58°C for 30 sec, followed by melting curve analysis consisting of 95°C for 15 sec, 60°C for 30 sec, and a final 95°C for 15 sec. β-actin expression was measured using certified Lux primers (Invitrogen) and used as a control for template loading in the real-time reactions. Real-time data were analyzed using Applied Biosystems 7300 Sequence Detection Software v1.3.1 (Applied Biosystems).

Immunofluorescence

For transient expression of pUL138, MRC5 fibroblasts were transfected using Amaxa nucleofection according to the manufacturer's instructions (Lonza, Gaithersburg, MD). Transfected cells were processed for immunofluorescence at 72 h post-transfection. For virus infection, MRC5 fibroblasts were seeded at 5x10^4 cells per well onto glass coverslips in 24 well plates and mock-infected or infected with either FIX-UL138_myc or FIXsubUL138 at an MOI of 2. Cells were washed in phosphate-buffered saline (PBS) (136.8 mM NaCl, 9.6 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4), fixed in 2% paraformaldehyde in PBS at room
temperature for 20 min, and permeabilized with 0.1% Triton X-100 in PBS for 15 min at room temperature. Cells were blocked in PBS containing 2.5 mg/ml BSA and 1% normal goat serum for 30 min at 4°C. Primary antibodies used are listed in Table 2. Secondary antibodies conjugated to fluorescent molecules included: Alexa Fluor 647-conjugated goat anti-mouse and Alexa Fluor 546-conjugated goat anti-rabbit IgG (H+L) (Invitrogen). Secondary antibodies were applied in PBS + 0.05% Tween-20 for 1 h at room temperature. Cells were washed with PBS + 0.05% Tween-20 and incubated in 1 μg/ml DAPI in PBS for 5 min at room temperature. Coverslips were mounted on slides with Aqua Polymount (Polysciences, Inc, Warrington, PA) and visualized using a Zeiss 510 Meta Confocal Microscope (Carl Zeiss Microimaging, Inc. Thornwood, NY). Images were re-colored artificially.

**Table 2 Primary Antibodies Used for Immunofluorescence and Immunoblotting.**

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aDilution in PBS supplemented with BSA and Tween 20; incubated for 60-90 minutes at room temperature or at 4°C overnight in the case of the rabbit anti-myc antibody.

bDilution in TBS supplemented with BSA and Tween 20; incubated for 60-90 minutes at room temperature.

cProvided by Lonnie Lybarger (150)

dGenerous gift from Tom Shenk, Princeton University

IF, immunofluorescence; IB, immunoblot, R, rabbit; M, mouse monoclonal; GM, Golgi matrix; GS, Golgi SNARE; PDI, protein disulfide isomerase; EEA1, early endosomal antigen 1; n/d, not done

**Microsomes**

A confluent 10 cm dish of ARPE-19 cells were transfected with 30 µg of Lipofectamine 2000 and 30 µg of pEF1-UL138\textsubscript{WT}. Crude membrane fractions were prepared by resuspending cells in buffer A (250 mM sucrose, 50 mM triethanolamine, 1 mM EDTA, 6 mM magnesium acetate, 50 mM potassium acetate, 1 mM DTT) at a density of 2x10\textsuperscript{7} cells per ml and sonicated 3 times for 10 seconds. Lysates were centrifuged at 3000xg for 5 min at 4°C. Supernatants and pellets were separated. Supernatants were centrifuged at 12,000xg for 10 min at 4°C. Supernatants and pellets were again separated and supernatants centrifuged at 25,000xg for 30 min. Pellets were resuspended in buffer A. Pellets and supernatants were analyzed by immunoblotting. The orientation of pUL138 in 25K light membrane fraction was determined by digesting intact microsomes with 0.5 ng/µl proteinase K for 30 min at 37°C in the presence or absence of 9 mM PMSF and analyzed by immunoblotting.
Microsomal membranes were salt stripped by resuspension in 100 mM Na$_2$CO$_3$, incubated on ice for 1 h, and pelleted at 50,000 RPM in a TLA-100.3 rotor (Beckman Coulter) at 4°C for 45 min. Stripped membranes were resuspended in Buffer A and analyzed by immunoblotting.

Purification of Extracellular Virions

Extracellular virions were isolated from cell-free tissue culture supernatants from FIXwt-infected MRC5 cells by density gradient centrifugation through a 20% D-sorbitol cushion for 80 min at 20,000 RPM in an SW28 rotor (Beckman Coulter) at 22°C. Virions were further purified by banding on glycerol-tartrate gradients as described previously (158). Briefly, virions were resuspended in 0.1 ml TN (50 mM Tris pH 7.4, 100 mM NaCl) and layered on top of a linear 9 ml glycerol-tartrate gradient formed in TN. Gradients were centrifuged at 34,000 RPM for 90 min at 22°C in an SW41 Ti rotor (Beckman Coulter) and then further centrifuged at 34,000 RPM for 18 h at 4°C. Virions were visualized using incandescent light, collected, diluted in 10 ml TN, and centrifuged at 34,000 RPM for 80 min at 22°C in an SW41 Ti rotor (Beckman Coulter). Pelleted particles were resuspended in 0.1 ml TN and analyzed by immunoblotting.
**Results**

*UL138 is encoded on two large transcripts during productive infection*

Using our experimental model for HCMV infection in HPCs, we have identified sequences in the ULb’ region of the genome, specifically the putative *UL138* ORF, that are required for a quiescent infection with the hallmarks of latency *in vitro* (53). To begin to characterize the gene products derived from the *UL138* locus, we analyzed transcription through this region in productively infected MRC5 fibroblasts by northern blotting. Total RNA was analyzed by northern blotting using a radiolabeled antisense ribonucleotide probe to the entire *UL138* coding sequence. Transcripts were detected at 2.7- and 3.6-kb (Fig. 4A) as early as 6 h post-infection and accumulated throughout the time course of infection. To characterize the viral kinetics of *UL138* transcription, cells were treated with cyclohexamide (CHX) or phosphonoacetic acid (PAA), inhibitors of translation and viral DNA replication, respectively. *UL138* transcript levels were somewhat reduced in the presence of PAA and undetectable in the presence of CHX. These results suggest that *UL138* expression in fibroblasts is dependent on the synthesis of the immediate early (IE) proteins. In contrast, *UL138* expression is enhanced by, but not dependent on, viral DNA synthesis. Taken together, these results suggest that *UL138* is expressed with early-late gene kinetics during productive infection in fibroblasts.
Figure 4 Analysis of UL138 transcripts. (A) Total RNA isolated from FIXwt-infected (MOI=2) MRC5 cells over a time course was analyzed by northern blotting using a radiolabeled antisense UL138-specific probe. To determine the kinetics of UL138 expression, cells were treated with cyclohexamide (CHX) or phosphonoacetic acid (PAA) and RNA was isolated at 24 hpi. (B) Poly-A+ purified RNA isolated from infected MRC5 cells at 24 hpi was analyzed by northern blotting using radiolabeled antisense probes specific to the ULb° ORFs indicated or a sense probe specific to UL138. The positions of the 3 and 4 kilobase RNA markers are shown to the left of each blot. (C) Schematic representation of 5' and 3' RACE results. Gene specific primers for 5' (gsp2) and 3' (gsp1) are shown by open arrows. The results shown in each panel are representative of at least three experiments.

The size of the UL138 transcripts was surprising given that the UL138 ORF is 510 nucleotides in length. To map the transcripts by northern blotting, we used radiolabeled antisense RNA probes to 3 ORFs upstream of UL138 (UL133, UL135, and UL136) and 2 ORFs downstream, newORF11 (100) (herein referred to as UL138b) and UL139 (Fig. 4B). Our analysis indicates that the poly-A+ purified UL138 transcripts expressed in fibroblasts likely share a common 3' end,
including sequences to *UL138b*. The 5' ends of the 2.7- and 3.6-kb transcripts include ORFs as far upstream as *UL135* and *UL133*, respectively. The antisense ribonucleotide probes to the ORFs upstream and downstream of *UL138* did not identify any other transcripts in addition to the 2.7- and 3.6-kb transcripts. Further, no transcripts were detected using a sense probe to the *UL138* coding sequence, indicating that transcription originates from a single strand in this region of the genome.

We precisely mapped the 5' and 3' termini of each transcript by rapid amplification of cDNA ends (RACE) using overlapping primers to *UL138* (Fig. 4C, open arrows). cDNA was derived from mock- or FIXwt-infected fibroblasts at 24 hpi. From these analyses, the 5' end of the 3.6-kb transcript is at nucleotide position 190476, 8 nucleotides upstream of the predicted start of *UL133*. The 5' end of the 2.7-kb transcript is at nucleotide position 189551, 24 nucleotides downstream of the predicted start of *UL135*. In multiple experiments, we detected a single 3' RACE product, indicating that both the 3.6- and 2.7-kb transcripts terminate at a polyadenylation site immediately downstream of *UL138b* (nucleotide 186833). The transcripts are illustrated schematically in Figure 4C. Using the 5' and 3' ends, we cloned and sequenced the *UL138* cDNAs. Both transcripts are co-linear with FIX genomic sequences, suggesting that these transcripts are not spliced.
**UL138** is transcribed with differential kinetics in fibroblasts and CD34⁺ HPCs

Using quantitative real-time reverse transcriptase PCR (qRT-PCR), we analyzed the expression kinetics of polyadenylated UL138 transcripts in fibroblasts and CD34⁺ HPCs infected *in vitro* with FIXwt. Transcripts were quantitated using a standard curve derived from PCR product for UL122 and UL138. β-actin expression levels were used as a control for template loading. In productively infected MRC5 cells, UL138 was expressed starting at early times post infection, consistent with our RNA analyses in Figure 1A. UL138 transcripts accumulated as the infection progressed to levels exceeding that of UL122 transcripts encoding the IE2-86-kDa protein (Fig. 5A).
A  MRC5

Copies per 5x10^3 Cells

Hours Post Infection

B  CD34+

Copies per 5x10^3 Cells

Days Post Infection
**Figure 5 Kinetics of UL138 Expression.** Total RNA was isolated from (A) MRC5 cells or (B) CD34+ HPCs over a time course following infection with FIXwt at an MOI of 2. cDNAs were detected by real-time reverse transcriptase PCR (qRT-PCR) using UL138- and UL122-specific D-Lux primers and quantified by comparison to standard curves generated from PCR amplified gene sequences. Reactions using RNA derived from mock-infected cells or infected cells where the reverse transcriptase was omitted (RT-) from the cDNA synthesis serve as negative controls. The black and gray bars represent data for UL138 and UL122, respectively. Bars represent the average of two experiments each performed in triplicate for panel A and the average of two experiments for panel B. The insets show real time ΔR_{n} curves for β-actin at each time point to demonstrate that template loading into each reaction was equivalent. Standard deviations are shown for the replicates in panel A. The brackets in panel B represent the range of the data points in the two experiments performed.

The trend of UL138 expression differed in HPCs infected with FIXwt (Fig. 5B). UL138 transcripts were detected at maximal levels immediately following infection but steadily decreased during the time course of infection. UL122 transcripts were detected at lower levels during early points of the time course compared to UL138. We have detected both UL122 and UL123 in infected HPCs previously using microarrays (53, 54). It is not known if UL122 and UL138 are expressed in the same cell or if UL122 and UL138 are expressed in mutually exclusive subpopulations of infected cells. However, we have not detected IE1-72KDa or IE2-86KDa proteins in HPCs by either immunoblot (Fig. 7C) or immunofluorescence (data not shown). Over the time course, both UL122 and UL138 transcripts decreased to levels that were undetectable by 15 dpi. This data is consistent with the previously observed loss of HCMV gene expression in HPCs infected *in vitro* (52). The loss of detectable UL138 expression may reflect a global silencing of HCMV transcription that is proposed to occur during the
establishment of latency (69, 101, 122). Alternatively, the loss of \textit{UL138} transcripts could also reflect a loss of viral genomes during cellular proliferation, a limitation of \textit{in vitro} culture of CD34$^+$ HPCs (52). Nevertheless, \textit{UL138} was detected in CD34$^+$ cells as well as CD14$^+$ monocytes derived from healthy seropositive individuals (53).

\textit{UL138} encodes a 21-kDa protein during productive infection

To determine if \textit{UL138} encodes a protein, we generated a rabbit polyclonal antiserum against a 19 amino acid peptide (MAVTAPLDVDLLKPVTGS) near the carboxyl terminus of the \textit{UL138} coding sequence (Fig 6A). The antiserum was affinity purified using the immunizing peptide. In addition, we engineered several recombinant viruses (Fig. 6B). \textit{FIX-UL138STOP} was engineered by substituting a stop codon (TAA) at codon 15 (ATG) of the \textit{UL138} coding sequence (Fig. 6B, FIX-\textit{UL138STOP}). To aid our investigation in the protein coding capacity of \textit{UL138}, we also constructed a recombinant virus that expresses \textit{UL138} with an in-frame, C-terminal myc epitope tag. Similar to the \textit{UL138-null} virus lacking the entire \textit{UL138} coding sequence (Fig. 6B, FIX\textit{subUL138}) (53), \textit{FIX-UL138STOP} and \textit{FIX-UL138myc} replicated similarly to the wild-type virus (Fig. 6C).
Figure 6 UL138 amino acid sequence and recombinant UL138 viruses. (A) Amino acid sequence of pUL138. The putative transmembrane domain is shaded and the immunizing peptide used to create the rabbit anti-UL138 antibody is underlined. (B) Schematic of the recombinant viruses: FIX$_{sub}$UL138, FIX-UL138$_{STOP}$, and FIX-UL138$_{myc}$. (C) Multi-step growth curve analysis of FIX recombinant viruses. MRC5 cells were infected at 0.01 TCID$_{50}$ per cell and virus titers measured by TCID$_{50}$ over the time course indicated. Values represent the average of two independent experiments.

Lysates derived from MRC5 cells infected with FIX$_{wt}$, FIX$_{sub}$UL138, or FIX-UL138$_{STOP}$ were analyzed by immunoblotting using the rabbit anti-UL138 antibody. In FIX$_{wt}$-infected cells, we detected a protein of approximately 21-kDa corresponding to the 170 amino acid coding sequence of UL138 (Fig. 7A). This protein was not detected in mock-infected cells or cells infected with either FIX$_{sub}$UL138 or FIX-UL138$_{STOP}$. The UL138 protein is detected by 6 hpi, coincident with IE1 and IE2 protein synthesis and continues to accumulate during the productive infection in MRC5 cells (Fig. 7B). We next analyzed lysates derived from infected (GFP$^+$) CD34$^+$ HPCs by immunoblotting to determine if pUL138 was synthesized during a non-productive infection resembling latency. Interestingly, the 21-kDa protein was detected at 2 (Fig. 7C), 4, and 8 (data not shown) days postinfection despite the small number of transcripts detected by qRT-PCR in HPCs (Fig. 5B). The IE proteins were not detected in infected HPCs.
**Figure 7 pUL138 expression.** (A) Protein lysates derived from mock-infected MRC5 cells or cells infected with FIX<sub>wt</sub>, FIX<sub>subUL138</sub> or FIX-UL138<sub>STOP</sub> at an MOI of 2 were analyzed by immunoblotting using rabbit anti-UL138 antisera (5484), identifying a 21-kDa protein corresponding to UL138. (B) Immunoblot analysis of the kinetics of pUL138 expression compared to the IE1 and IE2 proteins. Protein lysates were derived from mock-infected MRC5 cells or cells infected with FIX<sub>wt</sub> at an MOI of 2 over a time course and analyzed using rabbit anti-UL138 (5484) and mouse anti-IE1/2 (3H4) antibodies. α-tubulin levels were used as a control for loading; the identity of the 43-kDa band in the mock infection is unknown but specific to the α-tubulin antibody. (C) Protein lysates derived from MRC5 cells infected with FIX<sub>wt</sub> (positive control, far left lane), mock-infected CD34<sup>+</sup> HPCs or CD34<sup>+</sup> HPCs infected with FIX<sub>wt</sub> were analyzed by immunoblotting with rabbit anti-UL138 (5484) and anti-IE1/2 (3H4) antibodies. β-actin serves as a loading control.

To confirm that the 2.7- and 3.6-kb transcripts encoding pUL138 are stably synthesized in cells infected with FIX-UL138<sub>STOP</sub>, we analyzed RNA derived from MRC5 cells infected with FIX<sub>wt</sub> or FIX-UL138<sub>STOP</sub> and treated with actinomycin D at 16 hpi. FIX-UL138<sub>STOP</sub> synthesized both UL138 transcripts as in the FIX<sub>wt</sub> infection. Over the 36-hour time course, UL138 transcripts synthesized in both FIX<sub>wt</sub> and FIX-UL138<sub>STOP</sub>-infected cells were stable and did not exhibit significantly different rates of decay that could abrogate protein production (Fig. 8). These results indicate that the UL138 transcripts synthesized during FIX-UL138<sub>STOP</sub> infection, while stable, do not serve as template for pUL138 synthesis.
Figure 8 The UL138 transcripts synthesized in cells infected with FIX-UL138STOP are stable. Cells infected with the FIXwt (FIX) or FIX-UL138STOP (STOP) at an MOI of 2 were treated with 4 µg/ml actinomycin D at 16 h postinfection. Total RNA was isolated over a time course and analyzed by northern blotting using a radiolabeled antisense probe to UL138. 18S and 28S ribosomal RNAs for each blot are stained with methylene blue and serve as a loading control (lower two panels).

The UL138 protein is required for HCMV latency

Virus-coded RNAs and proteins have been identified and demonstrated to function in the latent programs of α- and γ-herpesviruses. Our previous studies demonstrated that sequences encoding UL138 were required for HCMV to establish and/or maintain latency in vitro (53). To determine if pUL138 is required for latency, we analyzed infectious centers formation in pure populations of
CD34+ HPCs infected with FIX<sub>wt</sub>, FIX<sub>(ur)sub2</sub> (lacking UL136-UL142) (53), FIX<sub>subUL138</sub>, or FIX-UL138<sub>STOP</sub>. After 12 days in long-term culture, 10,000 infected CD34+ cells or an equivalent cell lysate were transferred to each of 12-24 wells of a 96-well dish seeded with fibroblasts. The cell lysate is a critical control for distinguishing virus preformed during the culture period prior to reactivation from virus formed as a result of reactivation. The fractions of wells containing GFP-positive fibroblasts for both the reactivation and preformed virus control experiments were scored 20 days later. FIX<sub>wt</sub> exhibited a typical latency phenotype where the fraction of wells scoring GFP-positive was 5-fold greater than that of the cell lysate (P=0.004) (Fig. 9). By contrast the FIX<sub>(ur)sub2</sub> lacking a 5 kb of the ULb' region including UL138 exhibits a complete loss of latency phenotype (P=0.21) with equivalent fractions of GFP-positive wells for the reactivation and preformed virus control. FIX<sub>subUL138</sub> and FIX-UL138<sub>STOP</sub> exhibited partial loss of latency phenotypes. The differences between the fraction of GFP-positive wells prior to and following reactivation were 1.4- and 1.7-fold for FIX<sub>subUL138</sub> and FIX-UL138<sub>STOP</sub>, respectively. The Student’s t test values for FIX<sub>subUL138</sub> and FIX<sub>subUL138STOP</sub> are 0.03 and 0.09, respectively. The partial loss of latency phenotype exhibited in FIX-UL138<sub>STOP</sub> infection indicates a requirement for pUL138 in the latent infection in vitro. However, the incomplete nature of the loss of latency phenotype exhibited by FIX<sub>subUL138</sub> and FIX-UL138<sub>STOP</sub> compared to the complete loss of latency exhibited by FIX<sub>(ur)sub2</sub>
(53) suggests that the UL138 transcripts or other ORFs in this region may also contribute to the latent infection of HCMV in vitro.

**Figure 9** The UL138 protein is required for HCMV latency. Pure populations of HPCs infected with FIXwt, FIX(ur)sub2, FIXsubUL138, or FIX-UL138STOP at an MOI of 2 were isolated by FACS. Following 12 days in long-term culture, 10,000 infected CD34+ HPCs or an equivalent cell lysate (preformed virus) were seeded into each of 12-24 wells of a 96-well dish containing MRC5 cells in a cytokine-rich medium. Wells were scored at 20-24 dpi for GFP-positive fibroblasts. The fraction of wells scoring positive for virus production in the reactivation experiments (black bars) is compared to the preformed virus detected in cell lysates (gray bars) in 5-6, independent experiments for FIXwt, FIX(ur)sub2, and FIX-UL138STOP and three independent experiments for FIXsubUL138. The standard error of the means is shown. The $P$ values for the comparisons of the fraction of GFP-positive wells in the reactivation to the preformed virus are 0.004, 0.21, 0.03 and 0.09 for FIXwt, FIX(ur)sub2, FIXsubUL138 and FIX-UL138STOP, respectively.
**pUL138 does not down-regulate immediate early gene expression**

pUL138 might contribute to latency by down-regulating gene expression from the major immediate early promoter (MIEP) to suppress viral replication. Indeed, recombinant virus lacking *UL138* replicate to greater levels in infected HPCs (53), indicating greater IE gene expression. To determine if UL138 directly affects MIEP activity, the coding sequence for *UL138*, including its Kozak sequence, was cloned in frame into the pEF1/Myc-His expression vector, generating a promoter driven carboxy-terminal-myc-tagged-UL138 construct, pEF1-UL138\textsubscript{WT}. A similar vector, pEF1-UL138\textsubscript{STOP}, was constructed by cloning the *UL138* coding sequence containing a stop codon (Fig. 6B) to prevent protein expression. We performed dual luciferase assays to analyze the activity of the MIEP in the presence or absence of pUL138. U373 cells were transfected with increasing amounts of pEF1-UL138\textsubscript{WT} or pEF1-UL138\textsubscript{STOP} and a constant amount of the reporter vectors, pGL3-MIEP\textsubscript{1400} and pRL-SV40. pGL3-MIEP\textsubscript{1400} expresses the firefly luciferase gene from the complete MIEP including enhancer and modulator regions and pRL-SV40 expresses renilla luciferase from the SV40 early promoter. pEF1-UL138\textsubscript{STOP} serves as a negative control and produced results similar to a pEF1-EMPTY vector control (data not shown). At 48 h post transfection, firefly luciferase activity was measured and normalized to renilla luciferase activity to control for transfection efficiency. *UL138* expression did not affect the expression of luciferase from the MIEP regardless of dose (Fig. 10A). Expression of pUL138 was confirmed by immunoblotting (data not shown). This
experiment was repeated in other cell lines including HeLa, ARPE-19, and 293 cells with similar results (data not shown). Additionally, in experiments using only the enhancer region of the MIEP to drive luciferase expression, pUL138 also failed to repress MIEP activity (data not shown). These results provide evidence that pUL138 does not function by downregulating expression from the MIEP in the cells tested.
Figure 10 pUL138 does not suppress IE gene expression. (A) U373 cells were transfected with increasing amounts (0.1, 0.5, 1.0, 3.0 µg) of pEF1-UL138WT encoding UL138 or pEF1-UL138STOP encoding UL138 containing a stop codon substituted at codon 15 as a negative control. Cells were also transfected with 10 ng of pGL3-MIEP1400 expressing the firefly luciferase gene from the MIEP and 20 ng pRL-SV40 expressing renilla luciferase from the SV40 promoter as a control for transfection efficiency. (B) 293 cells were transfected with increasing amounts (0.1, 0.5, 1.0, 1.5 µg) of pEF1-UL138WT or pEF1-UL138STOP as described above. Cells were also transfected with 100 ng of pSVH-1 encoding both IE1 and IE2 driven from the MIEP in addition to pGL3-MIEP1400 (10 ng) and pRL-SV40 (20 ng). In both A and B, the total amount of DNA transfected was kept constant over the titrations using pEF1-EMPTY. Luciferase activity was measured at 48-72 h following transfection. (C) Protein lysates derived from FIXwt- or FIXsubUL138-infected (MOI=1) MRC5 fibroblasts were isolated over a time course following infection and analyzed by immunoblotting using rabbit anti-UL138 and mouse anti-IE1/2 (3H4), and mouse anti-α-tubulin as a loading control.

We next wanted to explore the possibility that pUL138 might modulate activity of the MIEP through interaction with the immediate early proteins (IE1 and IE2) in an inhibitory feedback mechanism to control immediate early gene expression. To determine if pUL138 requires IE1 and/or IE2 in order to alter MIEP expression, we expanded our dual luciferase assays to include an expression plasmid, pSVH-1, encoding both IE1-72kDa and IE2-86kDa proteins. We transfected 293 cells with increasing amounts of pEF1-UL138WT or pEF1-UL138STOP and constant amounts of pGL3-MIEP1400, pRL-SV40, and pSVH-1. Expression of IE1, IE2, and pUL138 in these experiments was confirmed by immunoblotting (data not shown). Firefly and renilla luciferase activities were quantified as described for Figure 10A. In the presence of the IE proteins, pUL138 failed to diminish transcriptional activation from the MIEP and, if anything, slightly induced activation in a dose dependent manner (Fig. 10B). Dual
luciferase assays were also performed using expression vectors encoding either IE1-72kDa or IE2-86kDa alone with similar results (data not shown). These results indicate that pUL138 does not repress expression from the MIEP even in the presence of IE1 and/or IE2 in the cells tested. Immunoblot analysis of lysates further revealed that pUL138 expression did not reduce the levels of IE1 or IE2 proteins in transfected cells (data not shown).

Finally, we analyzed IE1-72kDa and IE2-86kDa protein levels during infection in the presence or absence of pUL138. MRC5 cells were infected with FIXwt or FIXsubUL138 at an MOI of 1 and protein lysates were collected over a time course following infection and analyzed by immunoblotting. Consistent with reporter assays (Fig. 10A and B), UL138 expression did not suppress IE1 or IE2 expression in the context of a productive infection (Fig. 10C). Indeed, the expression of IE1-72kDa and IE2-86kDa protein was delayed and IE2-86kDa was expressed to lower levels in the FIXsubUL138 infection compared to the FIXwt infection. Delayed and diminished levels of IE1-72kDa and IE2-86kDa expression were observed in multiple experiments using independent virus preparations. Taken together, these data indicate that UL138 does not downregulate expression from the MIEP. However, our results do not address the possibility that UL138 alters IE1/2 function nor do they rule out the possibility that pUL138 affects the MIEP in a context-dependent manner.
**pUL138 localizes to the Golgi apparatus**

To determine the subcellular localization of pUL138, MRC5 fibroblasts were nucleofected with pEF1-UL138\textsubscript{WT} or empty vector and analyzed by indirect immunofluorescence using a rabbit anti-myc antibody. pUL138 localized to a discrete perinuclear compartment resembling the Golgi apparatus (Fig. 11A). Further analysis demonstrated that pUL138 co-localized with or was juxtaposed to the trans-Golgi/trans Golgi network (TGN) marker G58K, the medial-trans-Golgi marker GS27, and the cis-medial-Golgi marker GM130 during transient expression. The localization of pUL138 in the secretory pathway was specific to the Golgi as it did not colocalize with a marker for the endoplasmic reticulum (protein disulfide isomerase, PDI) or early endosomes (early endosomal antigen-1, EEA1). pUL138 localization to the Golgi is preserved in multiple cell types including APRE-19, HeLa, and 293 cells transiently expressing pUL138 (data not shown). We observe identical localization of the wild-type (non-epitope tagged) pUL138 in infection using our polyclonal antisera to pUL138, indicating that the myc epitope tag has not altered pUL138 localization. Localization of pUL138 to the Golgi is consistent with the presence of multiple Golgi localization motifs including three tyrosine sorting motifs (YXX\(\Phi\)) and a single acidic cluster dileucine motif (DXXLL), both of which have been shown to target herpesvirus proteins to the Golgi (4, 62, 84, 88).
Figure 11 pUL138 localizes to the Golgi apparatus. (A) MRC5 cells were nucleofected with the pEF1-UL138<sub>WT</sub> expression vector or empty vector (pEF1-EMPTY) as a negative control. pUL138 localization was visualized by indirect double-label immunofluorescence using a rabbit antibody (71D10) specific to the myc epitope. Cell structures were labeled using mouse monoclonal antibodies to the Golgi markers G58K, GM130 and GS27, to the endoplasmic reticulum marker, PDI, and the early endosomal marker, EEA1. In cells transfected with pEF1-EMPTY, the Golgi was marked using the GM130 Golgi marker. (B) MRC5 cells were mock-infected or infected with FIX-UL138<sub>myc</sub> or FIXsubUL138 at an MOI of 2. pUL138 was localized to the Golgi by indirect immunofluorescence over a time course using the rabbit anti-myc antibody (71D10) and the mouse anti-GM130 antibody. Cell nuclei are indicated by the DNA stain, DAPI. Localization was visualized by confocal microscopy.
The localization of pUL138 to the Golgi does not require other proteins specific to virus infection since Golgi localization occurs in the absence of viral infection. To determine if pUL138 localizes to the Golgi during viral infection, MRC5 cells were mock-infected or infected with FIX-UL138\textsubscript{myc} or FIX\textsubscript{sub}UL138 at an MOI of 2. Cells were fixed and stained by indirect immunofluorescence at 12, 24, 36, and 48 hpi using rabbit anti-myc and the GM130 Golgi marker. pUL138 was localized to the Golgi cisternae and juxtaposed with or colocalized to GM130 by 12 hpi (Fig. 11B) in a similar pattern as that observed in transient expression studies. The localization of pUL138 to the Golgi suggests that pUL138 may function by modulating cellular or viral processes mediated through the Golgi, such as viral egress, intracellular protein trafficking, and the cellular stress response. Further studies are underway to localize pUL138 in HPCs. While pUL138 is associated with membranes in HPCs, we have not yet demonstrated exclusive association of pUL138 with Golgi membranes (data not shown).

pUL138 is a type-1 integral membrane protein

To further analyze the Golgi association and subcellular distribution of pUL138 in infected cells, crude membrane fractions from FIX\textsubscript{wt}-infected MRC5 fibroblasts were prepared over a time course following infection and analyzed by immunoblotting for the presence of pUL138 using the rabbit anti-UL138 antibody and MHC class I (MHC-I) as a control using a mouse anti-MHC-I antibody
specific to the HLA-B/C haplotype. Golgi membranes are represented primarily in the microsomal (25K) fraction wherea the 3K and 12K pellets represent heavier membrane fractions containing cytomplasmic and nuclear membranes. Consistent with our localization studies (Fig. 11), pUL138 was detected primarily in the microsomal membrane fraction (25K pellet) by 12 hpi infection (Fig. 12A). Very low levels of pUL138 were present in heavier membrane fractions (3K and 12K pellets) or in the soluble fraction (25K supernatant). By contrast, MHC-I was present in all membrane fractions and the soluble fraction, indicative of its widespread distribution from the endoplasmic reticulum to the plasma membrane. The level of pUL138 in the microsomal membranes was stable over the time course. These data indicate that pUL138 is predominantly localized in the Golgi/microsomal membrane fractions at steady state. However, these data do not exclude the possibility that pUL138 cycles through the secretory pathway.
Figure 12 pUL138 is stably and predominantly localized to Golgi membranes. (A) Crude membrane fractions from FIXwt-infected (MOI=1) MRC5 cells were prepared over a time course following infection and analyzed by immunoblotting for the presence of pUL138 and MHC class I as a control. 25K pellets represent microsomal membrane fractions whereas 3K and 12 K pellets represent heavier membrane fractions. The 25K supernatant represents the soluble fraction. Unfractionated membranes from FIXwt- or mock-infected MRC5 cells (far left two lanes) served as positive and negative controls, respectively. Proteins were detected using the rabbit anti-UL138 antibody (5484) and a monoclonal antibody specific to the MHC class I antigens, HLA B/C. (B) To determine if pUL138 is an integral membrane protein, microsomal membranes were stripped with 100mM sodium carbonate, pelleted at 100,000xg and analyzed by immunoblotting for pUL138 and MHC-I using rabbit anti-UL138 and mouse anti-MHC-I antibodies, respectively. (C) To determine the orientation of pUL138 in Golgi membranes, microsomal membrane fractions were isolated from ARPE-19 cells 48 hours after transfection with pEF1-UL138\textsubscript{WT}. Microsomes untreated or treated with 0.5 ng/\mu l proteinase K, in the presence or absence of a protease inhibitor (9 mM PMSF), were analyzed by immunoblotting for pUL138 or MHC-I. pUL138 and MHC-I were detected using the rabbit anti-UL138 (5484) and mouse anti-MHC-I antibodies, respectively.

We next sought to determine if UL138 was an integral membrane protein in the Golgi and, if so, the orientation of the protein in the membranes. The predicted transmembrane domain of pUL138 is 3 amino acids from the N-terminus of the protein (Fig. 6A). To determine if pUL138 traversed the membrane, microsomal membrane preparations from infected cells were washed with salt and immunoblotted for MHC-I and pUL138 (Fig. 12B). The salt wash did not remove MHC-I from the membrane, as expected. Like MHC-I, pUL138 was stably associated with membranes. Further, pUL138 could not be solubilized from membranes with 1% digitonin, although stronger detergents such as 1% Triton X-100 or NP-40 will solubilize pUL138 (data not shown). Taken together, these results indicate that pUL138 is an integral membrane protein.
To determine the orientation of pUL138 in the Golgi membranes, microsomal membranes were isolated from ARPE-19 cells transfected with pEF1-UL138\textsubscript{WT}. Microsomes were subjected to proteinase K treatment in the presence or absence of a protease inhibitor. Untreated and treated microsomes, in addition to an infected cell lysate, were analyzed by immunoblotting for pUL138 and MHC-I as a control. Proteinase K digestion of the type-I membrane protein MHC-I in microsomes results in a smaller molecular weight cleavage product (Fig. 12C, second lane) due to cleavage of the short carboxy-terminal tail on the cytoplasmic face of the membranes. However, the large N-terminal domain of MHC-I is protected in the lumen of the Golgi/microsomes. Microsomal pUL138, however, was sensitive to proteinase K digestion. Given the position of the transmembrane domain (Fig. 6A), our results indicate that pUL138 is a type-I membrane protein where the large C-terminal domain of pUL138 is exposed on the cytoplasmic face of Golgi/microsomal membranes and is susceptible to proteinase K digestion.

**pUL138 is not incorporated into virus particles**

The localization of pUL138 in Golgi membranes prompted us to ask if virus particles acquire pUL138 during secondary envelopment and egress through the Golgi/endosomal compartments. If pUL138 is incorporated into virions, the large C-terminal cytoplasmic domain of pUL138 should extend into the tegument leaving only three amino acids on the surface of the virion (Fig.
13A). By contrast, lumenal proteins, such as viral glycoprotein B, are exposed on the surface of virus particles following egress (44). We analyzed virions purified on glycerol-tartrate gradients for the presence of pUL138 and the tegument protein pp71. By contrast to pp71, detection of pUL138 in untreated virion preparations was inconsistent, ranging from low to undetectable levels of protein. Two representative experiments are shown in Figure 13B.

The inconsistent detection of pUL138 relative to the consistent detection of pp71 led us to suspect that the pUL138 present in virion preparations may be a contaminant. Over 70 cellular proteins are reported to co-purify with virus particles and it is difficult to distinguish these proteins as bona fide virion components or co-purifying contaminants (171). MHC-I protein was analyzed in virus preparations as an indicator of contamination since this protein is present in Golgi membranes but not virus particles. We only detected pUL138 in virion preparations that also contained high levels of MHC-I (Fig. 13B, experiment 1).
Figure 13 pUL138 is a type-I membrane protein that is not packaged in the virus particle. (A) Schematic of secondary envelopment and egress of HCMV particles. A hypothetical virion particle illustrating the proposed orientation of pUL138 and MHC-I if these Golgi-localized proteins were incorporated into the virus envelope is shown. The chart illustrates the known sensitivities of MHC-I and pUL138 in microsomal membranes, the known sensitivity of pp71 in virions, and the proposed sensitivities of MHC-I and pUL138 in virions to trypsic digest. (B) Extracellular virions were purified by centrifugation through a sorbitol cushion followed by banding on glycerol-tartrate gradients. Virions were treated with trypsin at a 20:1 protease to protein ratio in the presence or absence of 1% Triton X-100. 5 µg of protein per lane was analyzed for the presence of pUL138, pp71, and MHC-I by immunoblotting using rabbit anti-UL138 (5484), mouse anti-pp71, and mouse anti-MHC-I antibodies, respectively. Two of four independent experiments are shown. A lysate of FIXwt-infected cells is included as a positive control.
To further test the possibility that pUL138 is a co-purifying contaminant in virion preparations, we analyzed the accessibility of pUL138 in purified virions to trypsin digestion in the presence or absence of detergent to disrupt the viral envelope (Fig. 13B). We further analyzed the viral pp71 tegument protein as a control for intact virions and MHC-I as a control for contaminating membrane proteins. The anticipated sensitivity or protection of these proteins to trypsic digest when associated with microsomes or virus particles is shown (Fig. 13A, chart). As expected, pp71 was protected from proteolytic digestion and became sensitive to digestion only in the presence of detergent. By contrast, pUL138 was susceptible to trypsin proteolysis in the absence of detergent in virion preparations when it was detected (Fig. 13B, Experiment 1), whereas MHC-I was susceptible to degradation only in the presence of detergent. Digestion of MHC-I to the lower molecular weight species observed in each experiment is consistent with published results analyzing trypsic digestion of membrane-associated MHC-I (112) and full digestion of MHC-I is not expected with trypsin. These results are consistent with the topology of pUL138 and MHC-I in microsomal membranes, but not virus particles (Fig. 13A). The inconsistent detection and the topology of pUL138 in virus preparations is consistent with the supposition that pUL138 and MHC-I are contaminants of virion preparations, perhaps arising from microsomes or exosomes associated with viral infection (3, 137). Based on these analyses, we conclude that it is unlikely that pUL138 is a constituent of the viral envelope. This finding implies that the function of pUL138 during the latent or productive
infection requires *de novo* synthesis of pUL138 as it is not delivered to the cell by the virion.

**Discussion**

UL138 is the first viral determinant demonstrated to function in promoting HCMV latency (53). *UL138* is a previously uncharacterized 510-nt gene encoded within the ULb' region of the genome unique to clinical or low passage stains of HCMV. Our study represents an initial characterization of the *UL138* gene products. During a productive infection, *UL138* is transcribed on two large transcripts expressed with early-late kinetics (Fig. 4). Expression of *UL138* is differentially regulated in infected fibroblasts and CD34+ HPCs (Fig. 5), sites of productive and latent infections, respectively. *UL138* gives rise to a 21-kDa (Fig. 7), type-1 transmembrane protein (Fig. 12) that localizes to the Golgi during viral infection (Fig. 11B) and in the absence of other viral proteins (Fig. 11A). Importantly, the UL138 protein is required for HCMV to efficiently establish and/or maintain a latent infection in CD34+ cells infected *in vitro* (Fig. 9). However, our results do not preclude a role for the *UL138* transcripts or other putative ORFs encoded on the UL138 transcripts (*UL133-UL138b*) or deleted in FIX(ur)sub2 (*UL136-UL142*). The function of pUL138 in infection is not realized upon viral entry, but requires *de novo* synthesis of pUL138, since pUL138 is not delivered to infected cells as a virion component (Fig. 13).
This work represents the first characterization of the HCMV latency determinant, UL138. While *UL138* is important to the latent infection in our *in vitro* system, other viral and cellular components likely exist that also contribute to the latent infection. Indeed, our data (comparing FIX(ur)sub2 and *UL138*-null viruses in Figure 9) suggests a requirement for other viral factors in establishing and maintaining the latent infection. The *UL138* transcripts as well as other potential ORFs in the ULb´ region may be required for HCMV latency. Future studies will decipher the mechanism by which *UL138* promotes the latent infection and identify additional viral or cellular determinants that contribute to HCMV latency.
CHAPTER 3: CLAMP INTERACTIONS

Introduction

We have shown that pUL138 is functionally required, but not sufficient for latency during in vitro infection of CD34+ hematopoietic progenitor cells (HPCs) (111). Based on this observation as well as the Golgi-localization and cytosolic accessibility of pUL138 in the membrane, we hypothesized that other viral and cellular factors likely interact with pUL138 and contribute to the latent infection. Our laboratory has identified 3 novel proteins pUL133, pUL135, and pUL136, that are encoded on the 3 large UL138 transcripts (55). We have named this novel genetic locus the CLAMP locus for HCMV Latency Associated Membrane Proteins (168).

We next wanted to know if, similar to pUL138, the 3 additional CLAMPs localize to the Golgi and if they interact with one another. To answer these questions we analyzed the subcellular localization of each of the CLAMPs both during infection and when expressed transiently in mammalian cells during infection with a recombinant HCMV strain lacking the CLAMP locus. Additionally, using a systematic immunoprecipitation analysis in transiently transfected cells, we analyzed each of the potential one-on-one interactions between the CLAMPs and subsequently identified definitive interactions between pUL133 and pUL138 and between pUL136 and pUL138. Further, we found that pUL133, pUL136 and pUL138 have the ability to self-associate.
To better understand the importance of these interactions and the role of this novel protein complex in infection, we focused on the most robust interaction, the interaction between pUL133 and pUL138. We mapped the interacting domains of pUL138 required for the interaction with pUL133 to a specific domain in the cytosolic tail of pUL138. While the function of this novel interaction remains unknown, this is the first report of another viral protein interacting with the HCMV latency determinant pUL138. Intriguingly, while we detect the interaction of pUL133 with pUL138 during infection in fibroblasts, we do not detect this interaction during infection in epithelial or endothelial cells (Personal Communication, Umashankar and Goodrum), suggesting that this interaction may be cell type-specific and may therefore play a role in regulating the outcome of infection.

**Materials and Methods**

**Cells**

Human embryonic kidney 293T cells (ATCC, Manassas, VA) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 100 U/mL penicillin, and 100 µg/mL streptomycin. Primary human embryonic lung fibroblasts (MRC5) (ATCC) were maintained as described previously (71). HeLa cells (ATCC) were cultured in DMEM supplemented with 10% normal calf serum (NCS), and 100 U/mL
penicillin, and 100 µg/mL streptomycin. All cells were maintained at 37°C with 5% CO₂.

Viruses

HCMV Strains

The recombinant HCMV strains FIX-UL133\textsubscript{myc}, FIX-UL135\textsubscript{myc}, FIX-UL136\textsubscript{myc}, and TB40E-Δ3.6 were described previously (55, 168). The recombinant HCMV strain FIX-UL138\textsubscript{myc} was described previously (Chapter 2, and (111)). The recombinant HCMV strain TB40E-SUPER was generated through a previously described multistep BAC recombineering protocol (178) (111) with several modifications. First, a large portion of the ULb’ region from UL133 through UL138 was exchanged for the GalK cassette by linear recombination in the first step of the recombination process. This was accomplished by PCR amplifying the galK cassette from pGalK (50 ng) (178) with 50 base pair homology arms to UL133 and UL138 using a forward primer with partial homology to UL133 and galK, and a reverse primer with partial homology to UL138 and galK (Table 3). This generated an ULb’ region knockout BAC intermediate lacking UL133-UL138.

Next, a large ~4.3 Kb portion of the ULb’ region containing UL148a through UL138b from the WT TB40E BAC was cloned into pGEMT\textsubscript{Easy} through a three step PCR and allelic exchange based process (127). First, the genomic segment containing UL148a-UL133 was PCR amplified using a forward primer
with an EcoRV site and a reverse primer terminating in a preexisting endogenous HindIII site (Table 3). Simultaneously, the genomic segment containing UL138-UL138b was PCR amplified using a forward primer with the same endogenous HindIII site and a reverse primer with an EcoRV site (Table 3). The two resulting PCR amplicons were annealed, extended, and used as template in a second round of PCR to amplify the newly annealed product containing UL148a-UL133 combined with UL138-UL138b using the UL148a, EcoRV containing primer and the UL138b, EcoRV containing primer. This new fragment was flanked by two EcoRV sites and contained an internal preexisting HindIII site. Next, the fragment was cloned into the EcoRV site of pGEMTeasy generating pGEMT-(UL148a-UL133)-HindIII-(UL138-UL138b). Lastly, this construct was linearized with HindIII and used for allelic exchange to rescue the remaining portion of the ULb’ region between UL133 and UL138 thus generating a vector containing the entire genomic locus from UL148a through UL138b, pGEMT-UL148a-UL138b.

Epitope tags were then added to each of the CLAMPs through 4 sequential rounds of Phusion Mutagenesis (Finnzymes) using 5’ phosphorylated reverse primers containing the Flag (3X- DYKDDDDK), HA (YPYDVPDYA), and myc (EQKLISEEDL), epitope tags as well as a phosphorylated reverse primer with tandem StrepII (WSHPQFEK), Glu-Glu (EYMPME) tags according to the manufacturer's instructions (Table 3). The quadruple tagged pGEMT-UL148a-UL138b was then digested with EcoRV to release the modified ULb’ insert and then used as a linear oligo in the second round of the recombineering process to
exchange the galK cassette for the modified ULb' region by linear recombination in SW105 E. coli. P0s were generated by electroporation of the purified BAC DNA into MRC5 fibroblasts along with a plasmid encoding the HCMV tegument protein pp71. Fourteen to twenty one days later, primary stocks were harvested by collecting the cells and media in (Iscov’s Modified Dulbecco’s Media, IMDM) + 1% BSA and storing the stocks at -80°C until use. Infecting MRC5 fibroblasts with the processed primary stock after brief sonication to break up virus aggregates and liberate cell-associated virus, allowed for propagation of secondary virus stocks. The supernatant containing virus from secondary infections were harvested 10-20 days post infection when cells show 100% cytopathic effects. Supernatants were sonicated for 3 pulses at power 6.0, 80% duty cycle on a Branson 450 Cup Sonicator (Branson Ultrasonics) prior to clearing the cellular debris at 3220 RCF for 15 minutes at room temperature. Virus containing supernatant was then loaded into SW28 tubes with a 20% D-sorbitol underlayed cushion and centrifuged at 21000 RPM at 18°C for 1 hour 20 minutes. Virus pellets were resuspended to 100X in IMDM + 2% BSA and stored at -80°C until use. HCMV strains were tittered by tissue culture infectious dose (TCID$_{50}$) on MRC5 fibroblasts using a modified TCID$_{50}$ assay described previously (54).
Lentiviruses

To generate lentiviruses expressing each of the tagged CLAMPs, HEK293FT cells were transfected with pCIG2 variants containing UL133<sub>Flag</sub>, UL135<sub>HA</sub>, UL136<sub>myc</sub>, or UL138<sub>EE</sub> in combination with the lentiviral packaging vectors pVSV-G, pLP1 and pLP2 (kind gifts from L. Lybarger) in a 3:1:1:1 ratio using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Supernatant containing lentivirus was harvested at 48 and 96 hours post transfection and pelleted at 3220 RCF for 15 minutes at 4°C to remove cellular debris. Lentivirus particles were pelleted for 2 hours at 17000 RPM in an SW28 rotor (Beckman Coulter) at 4°C. 100X virus pellets were resuspended in IMDM + 2% BSA for 1 hour on ice, pooled, made into aliquots, and stored at -80°C until use.

Plasmids

Plasmids for analysis of the pUL133:pUL138 Interaction

To generate pCIG2-UL133<sub>HA</sub>-IRES-EGFP we PCR amplified UL133 from the TB40E BAC isolate using a reverse primer with an HA epitope tag (YPYDVPDYA) (Table 3) and cloned it into the Nhel and EcoRV sites of pCIG2-IRES-EGFP described previously (55). Additionally, we generated pCIG2-UL138<sub>myc</sub>-IRES-EGFP by PCR amplifying UL138 from the TB40E BAC isolate using a reverse primer with a myc epitope tag (EQKLISEEDL) (Table 3) and cloned it into the Nhel and EcoRV sites of pCIG2-IRES-EGFP. pCIG2-IRES-
EGFP was further engineered to contain two additional restriction sites downstream of the eGFP to facilitate further modification. Briefly; NotI and AgeI sites were added to pCIG2-IRES-EGFP by Phusion Mutagenesis (Finnzymes) according to the manufacturers instructions using a 5' phosphorylated forward primer containing the NotI and AgeI restriction sites (Table 3). This modification created a pCIG2 vector lacking an IRES and a reporter. It was further modified to express several drug resistance genes in place of eGFP. To accomplish this, each drug resistance gene was PCR amplified using a forward primer with an AgeI site and a reverse primer with a NotI site (Table 3). Additionally we PCR amplified the EMCV IRES from pIRES-puro (Clonetech) using a forward primer with a BamHI site and a reverse primer with an AgeI site (Table 3). Each drug resistance gene was then ligated to the IRES and the IRES-drug resistance fragments were PCR amplified using the IRES forward primer and the corresponding drug resistance gene reverse primer. Each of these fragments was then cloned into the BamHI and NotI sites of the modified pCIG2. The puromycin resistance gene was PCR amplified from pIRES-puro (Clonetech) generating pCIG2-IRES-PURO. The hygromycin resistance gene was PCR amplified from the MIH vector (gift from L. Lybarger) generating pCIG2-IRES-HYGRO. The neomycin resistance gene was PCR amplified form pEF1α (Invitrogen) generating pCIG2-IRES-NEO and the bleomycin resistance gene was PCR amplified form the MIB vector (gift from L. Lybarger), generating pCIG2-IRES-BLEO. All constructs were confirmed by digestion and sequencing.
Table 3 Primers for Plasmids and Recombinant HCMV Strains.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Orientationa</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td><strong>pCIG2 Modification Primers</strong></td>
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<td>3ACCCGACTCTGGCTTCCATTTCGACTTGGCATATTTGTATTTCTTGAACCCGCGACGCTTGGCAGCAGATCGTGCT</td>
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*Note: The orientation of the primers indicates whether they are forward (FWD) or reverse (REV). Primers are designed for specific sequences within the constructs, facilitating the creation of recombinant plasmids and strains.*
### TB40E-SUPER ORF Tagging Primers

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### TB40E-SUPER Recombining Primers

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<td>REV</td>
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### UL138 C-Terminal Truncation Primers

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<td>pCIG2-UL138(1-85)myc</td>
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<td>ggggGATATCCGACAGATCCTCTGAGATGAGGTTTGTGAGGATAGTAGGTACC</td>
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</table>

*a* FWD, forward; REV, reverse  
*b* 3, 5' phosphorylation  
*c* Enzyme sites are underlined.  
*d* Extra nucleotides are in lowercase  
*e* Used for all UL138 Truncations
Plasmids for CLAMP Immunoprecipitation / Co-localization Studies

UL133\textsubscript{Flag} was PCR amplified from the FIX BAC using a reverse primer containing the 3X-Flag epitope tag (3X- DYKDDDDK) (Table 3) and cloning the amplicon into the Nhel and EcoRV sites of pCIG2-IRES-BLEO generating pCIG2-UL133\textsubscript{Flag}-IRES-BLEO. UL135\textsubscript{HA} was PCR amplified from the TB40E BAC using a reverse primer with the HA epitope tag (YPYDVPDYA) (Table 3) and cloning it into the Nhel and EcoRV sites of pCIG2-IRES-HYGRO generating pCIG2-UL135\textsubscript{HA}-IRES-HYGRO. UL136\textsubscript{myc} was PCR amplified from the TB40E BAC using a reverse primer that incorporated the myc epitope tag (EQKLISEEDL) (Table 3) and cloning the UL136\textsubscript{myc} fragment into the Nhel and EcoRV sites of pCIG2-IRES-NEO yielding pCIG2-UL136\textsubscript{myc}-IRES-NEO. Using the TB40E BAC as template and either a reverse primer with a Glu-Glu tag (EYMPME) (Table 3) or a reverse primer with tandem StrepII (WSHPQFEK) and Glu-Glu epitope tags (Table 3), UL138\textsubscript{EE} or UL138\textsubscript{SII-EE} were PCR amplified and cloned into the Nhel and EcoRV sites of pCIG2-IRES-PURO generating pCIG2-UL138\textsubscript{EE}-IRES-PURO or pCIG2-UL138\textsubscript{SII-EE}-IRES-PURO. All constructs were confirmed by digestion and sequencing.

Plasmids for Domain Swap Immunoprecipitation Studies

Chimeric pUL133 with the transmembrane domain of pUL135 was engineered through a two-step process using both Phusion Mutagenesis (Finnzymes) and traditional cloning methods. Using forward and reverse primers
each containing half of the pUL135 transmembrane domain (Table 3), the transmembrane domain of pUL135 was substituted for the pUL133 transmembrane domain in pCIG2-UL133StrepII-IRES-BLEO by Phusion Mutagenesis (Finnzymes) according to manufacturer’s instructions. Next, the UL133(UL135TM) chimera was amplified from pCIG2-UL133(UL135TM)StrepII-IRES-BLEO by PCR using a reverse primer containing the HA epitope tag (Table 3). The amplicon was then cloned into the Nhel and EcoRV sites of pCIG2-IRES-BLEO generating pCIG2-UL133(UL135TM)HA-IRES-BLEO.

Through a single round of Phusion Mutagenesis (Finnzymes) on pCIG2-UL135HA-IRES-HYGRO using a forward and reverse primer each containing half of the pUL133 transmembrane domain (Table 3), we generated chimeric pUL135 with the transmembrane domain of pUL133 (pCIG2-UL135(UL133TM)HA-IRES-HYGRO). All constructs were confirmed by digestion and sequencing.

**Plasmids for CLAMP Self-Association Studies**

pCIG2-UL138Flag-IRES-PURO was generated by PCR amplifying UL138 from the clinical HCMV strain TB40E BAC isolate using a reverse primer containing the 3X-Flag epitope tag (Table 3). The UL138Flag construct was cloned into the Nhel and EcoRV sites of pCIG2-IRES-PURO generating a 3X-Flag tagged UL138 expression construct. The lentiviral expression vector pCIG2-IRES-EGFP and its derivatives pCIG2-UL133myc-IRES-EGFP, pCIG2-UL135myc-IRES-EGFP, and pCIG2-UL136myc-IRES-EGFP used in this study were
described previously (55). All constructs were confirmed by digestion and sequencing.

**Plasmids for pUL138 C-terminal Truncations**

Wild type pUL138 with a C-terminal myc tag (EQKLISEEDL) and 4 pUL138 C-terminal truncation mutants were generated by PCR amplifying portions of UL138 from BAC DNA of the TB40E clinical HCMV strain using primers containing C-terminal myc tags (Table 3). The pUL138\textsubscript{myc} construct and the C-terminal truncations were cloned into the NheI and BamHI sites of pCIG2-IRES-EGFP creating pCIG2-138\textsubscript{myc-IRES-EGFP}, pCIG2-138\textsubscript{1-142myc-IRES-EGFP}, pCIG2-138\textsubscript{1-114myc-IRES-EGFP}, pCIG2-138\textsubscript{1-85myc-IRES-EGFP}, and pCIG2-138\textsubscript{1-55myc-IRES-EGFP}. All constructs were confirmed by digestion and sequencing.

**Immunofluorescence**

**Indirect Immunofluorescence**

24-36 hours prior to transfection, HeLa cells were seeded onto 12 mm glass coverslips in 24 well plates. For transfection, media was aspirated and cells were washed once in PBS. Cells were then incubated in 0.5 mL of OPTIMEM (Invitrogen) in preparation for transfection. Transfections were performed according to manufacturer’s instructions with a few modifications. Briefly; 0.5 μg of plasmid was mixed with 100 μL Optimem while 2 μL
Lipofectamine 2000 (Invitrogen) was mixed with 100 µL Optimem and both were incubated for 5 minutes at room temperature. DNA and Lipofectamine mixtures were combined and incubated for 30 minutes at room temperature prior to adding to cells. 6 hours post transfection, the media was replaced with normal growth media including antibiotics. 48-72 hours post transfection, cells were processed for immunofluorescence as follows: Cells were washed 3 times in PBS and fixed in 2% paraformaldehyde for 20 minutes at room temperature. Cells were again washed 3 times in PBS and permeabilized in PBS with 0.1% Triton X-100 (Sigma) for 10 minutes at room temperature. Cells were then washed 3 times in PBS prior to blocking for 30 minutes in PBS containing 2.5 mg/mL BSA and 1% normal goat serum (NGS). Cells were incubated with primary antibodies (Table 1) in PBS containing 0.05% Tween 20 (PBS-T) overnight at 4°C and rinsed 3 times with PBS-T prior to incubation with secondary antibodies in PBS-T. Secondary antibodies conjugated to fluorescent molecules included: Alexa Fluor 647-conjugated goat anti-mouse and Alexa Fluor 546-conjugated goat anti-rabbit IgG (H+L) (Invitrogen). Finally, coverslips were washed 3 times with PBS and nuclei were stained with 1 µg/mL DAPI (4′, 6′-diamidino-2-phenylindole) in PBS for 5 minutes at room temperature prior to a single rinse in PBS and mounting onto slides with Aqua Polymount (Polysciences, Inc. Warrington, PA). Slides were visualized using a Zeiss 510 Meta Confocal Microscope (Carl Zeiss Microimaging) and processed and recolored artificially using ImageJ (NIH).
Direct Immunofluorescence

MCR5 fibroblasts were seeded on to 12 mm glass coverslips in 24 well plates 24 hours prior to infection. Cells were infected with the recombinant HCMV strain TB40E-Δ3.6 described previously (168) at an MOI of 2. 6 hours after infection, the cells were transduced with lentiviruses expressing each of the uniquely tagged CLAMP proteins alone or in combination. 48 hours post infection; cells were harvested for direct immunofluorescence as described previously (3.2.4.1) with a few modifications. Cells were washed, fixed, permeabilized and blocked as described (3.2.4.1). Prior to use, primary antibodies to the various epitope tags were conjugated to amine derivatized Quantum Dots (Molecular Probes, Invitrogen) of 525 nm, 565 nm, 585 nm, and 625 nm emission wavelengths according to manufacturers instructions (Table 4). Primary antibodies were incubated in PBS-T overnight at 4°C. Post staining, cells were washed 3 times in PBS-T and nuclei stained with Qnuclear Deep Red Stain (Molecular Probes, Invitrogen) according to manufacturer’s instructions. Coverslips were mounted using Qmount Qdot mounting media (Invitrogen) according to manufacturer’s instructions. Cells were imaged using a Ziess 510 Meta Confocal Microscope (Carl Ziess Microimaging) and images were processed and recolored using ImageJ (NIH).
Immunoprecipitations

HEK-293T cells on 10cm dishes were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturers instructions with a few modifications. Cells were rinsed once with phosphate buffered saline (PBS) (136.8 mM NaCl, 9.6 mM Na$_2$HPO$_4$, 2.68 mM KCl, 1.47 mM KH$_2$PO$_4$) and incubated at 37°C in 8 mL Optimem. 6.93 µg of each uniquely tagged CLAMP (pUL133Flag, pUL135HA, pUL136myc, and pUL138SII-EE) expression plasmid was mixed in 500 µL of Optimem (Invitrogen) and 14.85 mL of Lipofectamine 2000 was mixed in 500 µL Optimem. Both mixtures were incubated at room temperature for 5 minutes and then combined and allowed to incubate at room temperature for 30 minutes before being added to the cells. 6 hours post transfection media was aspirated and replaced with normal growth media containing antibiotics. 48 hours post transfection cells were harvested by trypsinization and pelleted at 450 RCF for 6 minutes at room temperature. Supernatants were aspirated and cell pellets resuspended in PBS + 20 mM iodoacetamide (IAA) and pelleted a second time at 450 RCF for 6 minutes at room temperature. Supernatants were discarded and cell pellets flash frozen in liquid nitrogen and stored at -80°C.

One day prior to immunoprecipitation, antibodies were mixed with Protein G Plus Agarose (Pierce) and incubated overnight at 4°C. Immunoprecipitations were carried out as follows: 20 µl of settled agarose was washed 3 times with IP wash buffer (PBS + 0.1% NP40 + 20mM IAA) and mixed with respective immunoprecipitation antibodies (Table 4) and incubated overnight at 4°C. The
next day, cell pellets were lysed in IP lysis buffer (PBS + 1% Igepal CA-630 (NP-40), 20 mM IAA, 0.2mM PMSF, 1X HALT (Pierce), 1X EDTA), for 1 hour at 4°C vortexing every 10 minutes. Lysates were cleared of nuclear debris by centrifugation at 14000 RCF for 20 minutes at 4°C. Supernatants were mixed with Zysorbin (Invitrogen) at a final concentration of 0.25% and shaken for 30 minutes at 4°C. The Zysorbin was pelleted at 10000 RCF for 5 minutes at 4°C. Final protein concentrations were determined by BCA assay (Pierce) and 100 µg of each protein lysate was analyzed by immunoprecipitation. Immunoprecipitation supernatants were collected post incubation of antibody/bead conjugate with lysate by pelleting the antibody/bead conjugates at 10000 RCF for 2 minutes at 4°C. The supernatants were concentrated by Tricholoracetic Acid (TCA)/Acetone precipitation using 1 supernatant volume of TCA to 8 volumes of cold acetone for 3 hours at -80°C. Proteins were pelleted at 17000 RCF at 4°C for 30 minutes before washing once in cold acetone. Pellets were air dried for 10 minutes and resuspended in non-reducing sample loading buffer (0.0625 M Tris HCl + 2% SDS + 10% glycerol + 0.002% bromophenol blue) and boiled for 5 minutes prior to loading to gels. For comparisons, 100 µg of each lysate was also TCA/Acetone precipitated prior to gel loading.
Table 4 Antibodies for Immunofluorescence, Immunoprecipitation, and Immunoblotting.

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[^a]: diluted in PBS + BSA + Tween 20; incubated at 4°C overnight

[^b]: diluted in PBS + BSA + Tween 20 + 2% NGS; incubated at 4°C overnight

M, mouse; R, rabbit; IF, immunofluorescence; IP, immunoprecipitation; IB, immunoblot; GM, Golgi matrix; n/d, not done

**Immunoblotting**

Cell pellets were lysed in IP lysis buffer at 4°C for 1-hour vortexing every 10 minutes. Lysates were cleared of nuclear debris by centrifugation at 14000
RCF for 20 min at 4°C. Supernatants were then incubated with Zysorbin (Invitrogen) at a final concentration of 0.25% with shaking for 30 minutes at 4°C. Zysorbin was then cleared by centrifugation at 10000 RCF for 5 minutes at 4°C. Protein concentrations were determined by BCA assay (Pierce) and 100 µg of each protein lysate was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using NuPage (Invitrogen) gels and transferred to 0.45-mm polyvinylidene fluoride (immobilon-FL, Millipore, Billerica, MA) membranes. Membranes were blocked in Tris-buffered saline (TBS) (25mM Tris [pH 8.0], 137 mM NaCl, 3 mM KCl, 1.5 mM MgCl2, pH 8.0) plus 5% nonfat dry milk and 2.5 mg/mL BSA for 1 hour at 4°C. Membranes were incubated in primary antibodies in TBS with 2.5 mg/mL BSA and 0.05% Tween 20 (TBS-BT) overnight at 4°C. Membranes were then washed 3 times for 15 minutes each in TBS-BT at room temperature prior to incubation with either goat-mouse IgG (H+L) Dylight 680/800-conjugated or goat anti-rabbit IgG(H+L) DyLight 680/800-conjugated secondary antibodies (Pierce). Membranes were then washed 3 times for 15 minutes each in TBS-BT and imaged using the Odyssey infrared imaging system (Li-Cor).

**Results**

**The CLAMPs localize to the Golgi during productive infection**

Having identified 3 additional proteins encoded on the large UL138 transcripts (55) (termed CMV Latency Associated Membrane Proteins, CLAMPs)
we wanted to determine the subcellular localization of these proteins during infection. To answer this question we previously generated recombinant viruses with C-terminal myc tags on UL133, UL135, or UL136 in the FIX clinical HCMV strain (55). Each of these viruses grew with similar kinetics in multistep growth curves (168) and expressed their respective myc-tagged ORF during productive infection in MRC5 fibroblasts. Additionally, each of these proteins was enriched in the Golgi-containing membrane fractions from crude membrane fractionations of infected fibroblasts (168). To corroborate the membrane fractionation we localized each of these novel proteins in MRC fibroblasts during productive infection. MRC5 cells were mock-infected or infected at an MOI of 2 with FIX-UL133\textsubscript{myc}, FIX-UL135\textsubscript{myc}, FIX-UL136\textsubscript{myc} or FIX-UL138\textsubscript{myc} and analyzed by indirect immunofluorescence 24 and 48 hours post infection. At 24 hours post infection, pUL133\textsubscript{myc} co-localized with the cis-Golgi marker GM130 but also localized to the cell membrane (Fig. 14). By 48 hours post infection, pUL133\textsubscript{myc} showed strong co-localization with GM130 suggesting that this protein may traffic during infection (Fig. 14). Like UL133\textsubscript{myc}, pUL135\textsubscript{myc} showed a similar localization pattern with both cell membrane and Golgi localization at 24 hours post-infection, and increased Golgi localization at 48 hours post-infection (Fig. 14). Similar to the localization of pUL138\textsubscript{myc}, pUL136\textsubscript{myc} consistently localized to the Golgi at both 24 and 48 hours post-infection (Fig. 14). All 4 CLAMPs localize to the Golgi by 48 hours post-infection suggesting that these proteins may cooperate to
modulate the outcome of infection from the Golgi through a novel mechanism involving Golgi functions.
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Figure 14 The CLAMPs localize to the Golgi during productive infection. MRC5 fibroblasts were mock infected or infected with FIX-UL133\textsubscript{myc}, FIX-UL135\textsubscript{myc}, FIX-UL136\textsubscript{myc} or FIX-UL138\textsubscript{myc} at an MOI of 2. Cells were analyzed by indirect double-label immunofluorescence at 24 or 48 hpi using an anti-myc antibody and a mouse antibody to the cis-Golgi marker GM130 (red). Nuclei are stained with the DNA stain, DAPI (blue). The localization of the indicated C-terminal myc-tagged ORF is shown (green). Co-localization is shown at right (yellow).

The CLAMPs co-localize when expressed exogenously in the context of infection

Since all 4 CLAMPs appear Golgi localized during infection we next wanted to know if these proteins co-localized in the same subcellular compartment. To answer this question generated lentivirus expression vectors where each CLAMP was cloned with a unique C-terminal epitope tag (pUL133\textsubscript{Flag}, pUL135\textsubscript{HA}, pUL136\textsubscript{myc}, and pUL138\textsubscript{EE}). We infected MRC5 fibroblasts with TB40E-Δ3.6, a recombinant HCMV strain lacking the entire CLAMP locus, and subsequently transduced the cells with lentiviruses expressing each uniquely tagged CLAMP either alone or in combination. We then analyzed the subcellular distribution of the CLAMPs at 48 hours post-infection by direct immunofluorescence using antibodies to each epitope tag conjugated to Quantum Dots. pUL133\textsubscript{Flag} and pUL135\textsubscript{HA} showed both a cell surface and perinuclear localization resembling the Golgi apparatus when expressed individually (Fig. 15). pUL136\textsubscript{myc} and pUL138\textsubscript{EE} both localize to a similar Golgi-like perinuclear compartment when expressed individually (Fig. 15). Importantly, when all 4 proteins are expressed together the localization of each
protein appears unchanged compared to when each protein is expressed individually (Fig. 15); pUL133Flag and pUL135HA show both a cell surface and Golgi-like localization while pUL136myc and pUL138EE show good localization in the same perinuclear Golgi-like compartment (Fig. 15). Importantly, we have shown previously that pUL138 is required for HCMV latency but likely involves the cooperation of additional viral factors encoded within the UL138 transcripts (55, 111). Taken together, these data indicate that pUL138 and the 3 additional CLAMPs co-localize and suggest that they might work together from the Golgi to modulate the outcome of infection through a novel mechanism.

The CLAMPs interact when expressed transiently

We have shown that each protein localizes to the Golgi during productive infection and that all 4 CLAMPs partially co-localize in a perinuclear compartment resembling the Golgi when exogenously expressed simultaneously during infection with a recombinant HCMV strain lacking the entire CLAMP locus. These observations prompted us to ask if the CLAMPs interact with each other. We began our search for interacting viral proteins by performing an extensive immunoprecipitation analysis of each of the potential one-on-one interactions between the 4 CLAMPs during transient expression in HEK293T cells.
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TB40EΔ3.6 + (UL133-8)
**Figure 15** The CLAMPs co-localize in the Golgi when expressed transiently during HCMV infection. MRC5 fibroblasts were mock infected or infected with TB40E-Δ3.6 at an MOI of 2 and subsequently transduced with lentiviruses expressing pUL133\textsubscript{Flag}, pUL135\textsubscript{HA}, pUL136\textsubscript{myc}, and pUL138\textsubscript{EE} alone or in combination. Cells were analyzed by direct immunofluorescence at 48 hpi using primary antibody Quantum Dot conjugates specific for the Flag, myc, HA, and Glu-Glu epitope tags. The localization of the indicated CLAMPs are shown; pUL135\textsubscript{HA} (Green), pUL136\textsubscript{myc} (RED), pUL133\textsubscript{Flag} (Orange) pUL138\textsubscript{EE} (Purple), Co-localization is shown at right. All images are digitally zoomed 1.7 X.

When we performed immunoprecipitations from lysates containing pUL133\textsubscript{Flag} and pUL135\textsubscript{HA} using rabbit antibodies to the Flag and HA epitope tags respectively, precipitation of pUL133\textsubscript{Flag} did not co-precipitate pUL135\textsubscript{HA} and precipitation of pUL135\textsubscript{HA} did not co-precipitate pUL133\textsubscript{Flag} (Fig. 16A). Importantly, immunoprecipitations without antibodies failed to precipitate any proteins (Fig. 16A). These results suggested that pUL133 and pUL135 do not interact in mammalian cells when expressed transiently.
Figure 16 One on one CLAMP interactions. (A-F) HEK293T cells were transfected with various combinations of uniquely tagged CLAMPs and assayed for protein-protein interactions by co-immunoprecipitation using rabbit α-Flag, rabbit α-HA, rabbit α-myc, and rabbit α-Glu-Glu antibodies followed by immunoblot using mouse α-Flag, mouse α-HA, mouse α-myc, and rabbit α-Glu-Glu antibodies. No antibody immunoprecipitations serve as negative controls.

Immunoprecipitation analysis with the Flag antibody from lysates containing pUL133Flag and pUL136myc failed to precipitate pUL136myc (Fig. 16B). Similarly, precipitation of pUL136myc with an antibody to the myc epitope tag failed to precipitate pUL133Flag (Fig. 16B). The lack of clear interactions suggested that pUL133 and pUL136 do not interact directly when expressed transiently in HEK293T cells. Interestingly, immunoprecipitations from lysates containing both pUL133Flag and pUL138StrepII-EE using the rabbit anti Flag antibody not only precipitated pUL133Flag but also co-precipitated pUL138StrepII-EE (Fig. 16C). Further, immunoprecipitation of pUL138StrepII-EE using the rabbit antibody to
the Glu-Glu epitope tag co-precipitated pUL133\textsubscript{Flag} (Fig. 16C). Taken together these results indicate that pUL133 and pUL138 interact in mammalian cells.

Immunoprecipitations with the antibody to the HA epitope tag from lysates containing pUL135\textsubscript{HA} and pUL138\textsubscript{SII-EE} pull down a small amount of pUL138 (Fig. 16D). However, immunoprecipitations in the opposite direction (using the Glu-Glu antibody) failed to precipitate pUL135 (Fig. 16D) suggesting that pUL135 and pUL138 may interact but weakly. Additional immunoprecipitations from lysates containing pUL135\textsubscript{HA} and pUL136\textsubscript{myc} hint that pUL135 and pUL136 might interact, albeit weakly (Fig. 16E). Immunoprecipitation using the antibody to the HA epitope tag pulled down a very small amount of pUL136, but immunoprecipitation using the antibody to the myc epitope tag failed to pull down pUL135 (Fig. 16E)

Lastly, precipitations from lysates containing pUL136\textsubscript{myc} and pUL138\textsubscript{Strepl-EE} using antibodies to the myc and Glu-Glu epitope tags co-precipitated small amounts of the corresponding partner (Fig. 16F) suggesting that these two proteins interact in mammalian cells. As a whole, these data indicate that several of the CLAMPs interact in mammalian cells when expressed transiently and suggest that the CLAMPs have the potential to interact during viral infection.

The CLAMPS Self-Associate

Having identified several interactions between the CLAMPs we next asked if the CLAMPs had the ability to self-associate. To answer this question, we
generated several uniquely tagged versions of each CLAMP and screened them for their ability to self-associate by immunoprecipitation followed by immunoblot. We transiently transfected HEK293T cells with two uniquely tagged pUL133 constructs (pUL133<sub>HA</sub> and pUL133<sub>myc</sub>), 2 uniquely tagged pUL135 constructs (pUL135<sub>HA</sub> and pUL135<sub>myc</sub>), 2 uniquely tagged pUL136 constructs (pUL136<sub>HA</sub> and pUL136<sub>myc</sub>) or 2 uniquely tagged pUL138 constructs (pUL138<sub>myc</sub> and pUL138<sub>Flag</sub>) and analyzed the lysates at 48 hours post transfection by immunoprecipitation followed by immunoblot. Immunoprecipitations using either the rabbit anti-myc or rabbit anti-HA antibodies from lysates containing 2 uniquely tagged pUL133 variants precipitated both pUL133<sub>HA</sub> and pUL133<sub>myc</sub> in either direction (Fig. 17A) suggesting that pUL133 has the ability to self-associate. Similarly, immunoprecipitations with antibodies to the myc and HA epitope tags from lysates containing two uniquely tagged variants of pUL136 (pUL136<sub>HA</sub> and pUL136<sub>myc</sub>) precipitated both tagged versions of pUL136 in either direction (Fig. 17C) suggesting that pUL136 has the ability to self-associate as well. Further, immunoprecipitations from lysates containing pUL138<sub>myc</sub> and pUL138<sub>Flag</sub> using the rabbit anti-myc or rabbit anti-Flag antibodies precipitated both tagged versions of pUL138 in either direction (Fig. 17D) arguing that pUL138 can self-associate as well. However, unlike the other CLAMPs, precipitations of pUL135 using the antibody to the myc epitope tag, only precipitated the myc-tagged version of pUL135 (pUL135<sub>myc</sub>) and failed to co-precipitate pUL135<sub>HA</sub> (Fig. 17B). Interestingly, immunoprecipitations using the antibody to the HA epitope tag,
precipitated both pUL135_{HA} and pUL135_{myc} suggesting that pUL135 may indeed self-associate but the interaction may not be as strong as that of the other CLAMPs. Taken together, the CLAMPs are not only able to interact with each other, but they are also able to self-associate suggesting the possibility that the CLAMPs might form a complex protein structure involving not only heteromeric interactions but also homomeric interactions.

**Figure 17 CLAMPs self-associate.** (A-D) HEK293T cells were transiently transfected with sets of 2 uniquely tagged versions of each of the 4 CLAMPs and screened for their ability to self-associate by co-immunoprecipitation using rabbit antibodies to the myc, HA, Flag, and Glu-Glu epitope tags, followed by immunoblot using mouse antibodies to the myc, HA, and Flag epitope tags, and rabbit antibody to the Glu-Glu epitope tag.
pUL133 and pUL138 interact when expressed transiently

From the one-on-one CLAMP interaction studies, the interaction between pUL133 and pUL138 was the most robust interaction, hinting at the potential importance of this interaction to the outcome of viral infection, specifically in relation to the established role of pUL138 in promoting viral latency (111). In an effort to better characterize this interaction we analyzed the interaction from under varying conditions by immunoprecipitation. First, we transiently transfected HEK293T cells with plasmids expressing pUL133\textsubscript{HA} and pUL138\textsubscript{myc} and analyzed the cell lysates by co-immunoprecipitation using the rabbit anti-myc or rabbit anti-HA epitope tag antibodies followed by immunoblot with mouse antibodies to the myc and HA epitope tags.
Figure 18 pUL133 specifically interacts with pUL138. HEK293T cells were transiently transfected with pUL133<sub>HA</sub> and pUL138<sub>myc</sub> alone or in combination and screened for protein-protein interactions under various conditions by co-immunoprecipitation using rabbit antibodies to the HA and myc epitope tags followed by immunoblot with mouse antibodies to the HA and myc epitope tags. Row 1) pUL138<sub>myc</sub> + pUL133<sub>HA</sub>, lysate containing both pUL138<sub>myc</sub> and pUL133<sub>HA</sub>. Row 2) +SDS, lysate containing both pUL138<sub>myc</sub> and pUL133<sub>HA</sub> were boiled in 1% SDS prior to immunoprecipitation. Row 3) Mixed Lysates, a lysate containing only pUL133<sub>HA</sub> combined with a lysate containing only pUL138<sub>myc</sub>. Row 4) pUL133<sub>HA</sub> alone, lysate containing only pUL133<sub>HA</sub>. Row 5) pUL138<sub>myc</sub> alone, lysate containing only pUL138<sub>myc</sub>.

In lysates containing pUL133<sub>HA</sub> and pUL138<sub>myc</sub>, co-immunoprecipitation with the rabbit antibody to the myc epitope tag not only cleared the supernatant and precipitated pUL138<sub>myc</sub> (Fig. 18, row 1, left panel, lanes 2 and 3), but also precipitated pUL133<sub>HA</sub> (Fig. 18, row 1, right panel, lane 3). Similarly, co-immunoprecipitation with the rabbit antibody to the HA epitope tag completely cleared the supernatant and precipitated all of the pUL133<sub>HA</sub> (Fig. 18, row 1, right panel, lanes 4 and 5) and also co-precipitated pUL138<sub>myc</sub> (Fig. 18, row 1, left panel, lane 5). A no antibody immunoprecipitation control failed to precipitate either protein (Fig. 18, top row, left panel, lane 7, and right panel, lane 7). These results demonstrate a specific interaction between pUL133 and pUL138 and confirm the interactions previously observed with alternatively tagged proteins. In most immunoprecipitations, the supernatants were completely cleared of protein (Fig. 18, row 1, left and right panels, lanes 2 and 4), but not in the case of the no antibody controls (Fig. 18, row 1, left and right panels, lane 7).

Importantly, immunoprecipitations from lysates containing both pUL133<sub>HA</sub> and pUL138<sub>myc</sub> which were boiled in 1% SDS prior to co-
immunoprecipitation precipitated only the target of each antibody and failed to precipitate the interacting partner (Fig. 18, row 2, left panel, lanes 3 and 5, and right panel, lanes 3 and 5). Additionally, immunoprecipitations from a mixture of two lysates, one containing only pUL133\(_{HA}\) combined with one containing only pUL138\(_{myc}\), precipitated only the targets of each antibody and failed to precipitate interacting partners (Fig. 28, row 3, left panel, lanes 3 and 5, and right panel, lanes 3 and 5). Importantly, to demonstrate specificity, immunoprecipitation with the rabbit antibody to the myc epitope tag from lysates containing only pUL133\(_{HA}\) failed to pull down pUL133\(_{HA}\) (Fig. 18, row 4). Conversely, immunoprecipitations with the rabbit antibody to the HA epitope tag from lysates containing only pUL138\(_{myc}\) failed to pull down pUL138\(_{myc}\) (Fig. 18, row 5). As a control for the antibodies used in immunoblotting, lysates containing each protein are run in lane 8 of each panel (Fig. 18, rows 4 and 5). Taken together, these data suggested that pUL133 and pUL138 specifically interact in mammalian cells.

**Distinct domains of pUL138 are required for interaction with pUL133**

Having demonstrated that pUL133 and pUL138 specifically interact in mammalian cells, we next wanted to map the interacting domains of pUL138 required for the interaction with pUL133. To further map the interacting protein domains of pUL138, 4 carboxy-terminal pUL138 truncations containing myc-tags, were PCR amplified from the TB40E BAC and cloned into pCIG2-IRES-EGFP generating 4 pCIG2-UL138\(_{myc}\)-IRES-EGFP variants; pCIG2-UL138\(_{1-142}\)myc-IRES-
EGFP, pCIG2-UL138\textsubscript{(1-114)myc-IRES-EGFP}, pCIG2-UL138\textsubscript{(1-85)myc-IRES-EGFP}, and pCIG2-UL138\textsubscript{(1-55)myc-IRES-EGFP} (Fig. 19). For each construct, the N-terminal portion of the protein containing the transmembrane domain of pUL138 was left intact to favor stability and proper localization of the variant proteins. To confirm protein expression, HEK293T cells were transiently transfected with each construct and cell lysates were harvested 48 hours post transfection and analyzed by immunoblot using the mouse anti-myc antibody. Each C-terminal pUL138 truncation expressed a myc-tagged pUL138 protein of the expected size (Fig. 20). It should be noted that pUL138 C-terminal truncations lacking amino acids 86-169 seem less abundant at steady-state perhaps due to a decrease in protein stability.
**Figure 19 pUL138 C-terminal truncations.** Schematic of wild type pUL138 and 4 pUL138 carboxy terminal truncations lacking different portions of the cytosolic tail of pUL138; pUL138(1-142)myc, pUL138(1-114)myc, pUL138(1-85)myc, and pUL138(1-55)myc. Each pUL138 truncation retains the pUL138 amino terminal portion including the transmembrane domain and also encodes a carboxy terminal myc epitope tag.

The subcellular distribution of each truncated pUL138 variant was analyzed by immunofluorescence to compare their distribution relative to the full-length pUL138. To analyze the localization of the C-terminal pUL138 truncations, we transiently transfected HeLa cells with each pUL138 truncation and analyzed the subcellular localization by indirect immunofluorescence.

![Short Exposure](image1)

![Long Exposure](image2)

**Figure 20 The pUL138 C-terminal truncations produce proteins.** HEK293T cells were transiently transfected with each of the pUL138 C-terminal truncations; pUL138(1-142)myc, pUL138(1-114)myc, pUL138(1-85)myc, and pUL138(1-55)myc and lysates were analyzed by immunoblot using a mouse antibody to the myc epitope tag with both a short and a long exposure. α tubulin is included as a protein loading control.
The pUL138 C-terminal truncations lacking amino acids 143-169 (pUL138(1-142)myc) and 115-169 (pUL138(1-114)myc) localize with the GM130 cis-Golgi marker in a pattern indistinguishable from the full-length protein (Fig. 21). While removal of amino acids 86-169 (pUL138(1-85)myc) resulted in localization of the pUL138 variant to the Golgi, pUL138(1-85)myc exhibited more diffuse staining around the Golgi compartment (Fig. 21). Further, truncation of amino acids 56-169 of pUL138 (pUL138(1-55)myc) resulted in increased diffuse staining with reduced GM130 co-localization (Fig. 21). The pUL138(1-55)myc variant is clearly detected in transfected cells by immunofluorescence (Fig. 21) despite the reduced levels of expression detected by immunoblotting (Fig. 20). All 4 of the pUL138 C-terminal truncations produced proteins that localized, at least in part, to the Golgi compartment and were, therefore, deemed suitable for mapping domains interacting with pUL133_HA.
Figure 21 pUL138 C-terminal truncation localization. HeLa cells were mock transfected or transfected with pUL138\textsubscript{myc}, pUL138\textsubscript{(1-142)myc}, pUL138\textsubscript{(1-114)myc}, pUL138\textsubscript{(1-85)myc}, or pUL138\textsubscript{(1-55)myc}. The localization of each pUL138 variant was determined by indirect double-label immunofluorescence using a rabbit antibody specific to the myc epitope and a mouse antibody to the cis-Golgi maker GM130 (red). Cell nuclei are indicated by the DNA stain, DAPI (blue). The localization of each myc-tagged, pUL138 C-terminal truncation is shown (green). Co-localization is shown at right (yellow).

To identify the pUL138 domains required for the interaction with pUL133, we transfected HEK293T cells with combinations of pUL133\textsubscript{HA} and each of the C-terminal truncations of pUL138 and analyzed the lysates 48 hours post-
transfection by immunoprecipitation followed by immunoblot. Immunoprecipitations were carried out using the rabbit anti-myc antibody to precipitate pUL138\textsubscript{myc}, or the rabbit anti-HA antibody to precipitate pUL133\textsubscript{HA}. Interacting proteins were detected using the mouse antibody to the myc epitope tag, or the mouse antibody to the HA epitope tag. As demonstrated previously (Figs. 16C and 18), the full-length pUL138 interacted with pUL133 and was efficiently immunoprecipitated from lysates in either direction using the myc or HA antibodies (Fig. 22, row 1, left and right panels, lanes 3 and 5). In lysates containing the pUL138 truncation lacking amino acids 143-169 (pUL138\textsubscript{(1-142)myc}) or 115-169 (pUL138\textsubscript{(1-114)myc}) (Fig. 22, rows 2 and 3), immunoprecipitation with the rabbit anti-myc antibody not only precipitated the pUL138 truncation (Fig. 22, rows 2 and 3, left panel, lane 3), but also co-precipitated pUL133\textsubscript{HA} (Fig. 22, rows 2 and 3, right panel, lane 3). Additionally, immunoprecipitation of pUL133\textsubscript{HA} with the rabbit anti-HA antibody from the same lysates not only precipitated pUL133\textsubscript{HA} (Fig. 22, rows 2 and 3, right panel, lane 5), but also precipitated pUL138\textsubscript{(1-142)myc} (Fig. 22, row 2, left panel, lane 5) and pUL138\textsubscript{(1-114)myc} (Fig. 22, row 3, left panel, lane 5). Further truncation of the pUL138 C-terminus including amino acids 86-169 (pUL138\textsubscript{(1-85)myc}) and amino acids 56-169 (pUL138\textsubscript{(1-55)myc}) resulted in complete loss of interaction with pUL133\textsubscript{HA}, as indicated by lack of co-immunoprecipitated proteins in either direction (Fig. 22, rows 4 and 5). These data suggest that a specific domain of pUL138 including amino acids 86-115 may be important for the interaction of pUL138 with pUL133.
Figure 22 Specific domains of pUL138 interact with pUL133. HEK293T cells were transfected with pUL133\textsubscript{HA}, in combination with pUL138\textsubscript{myc}, pUL138\textsubscript{(1-142)myc}, pUL138\textsubscript{(1-114)myc}, pUL138\textsubscript{(1-85)myc}, or pUL138\textsubscript{(1-55)myc}, and screened for protein-protein interactions by co-immunoprecipitation followed by immunoblot. Immunoprecipitations utilized rabbit antibodies to the myc and HA epitope tags, while immunoblots utilized mouse antibodies to the myc and HA epitope tags. Row 1) pUL138\textsubscript{myc} + pUL133\textsubscript{HA}, lysate containing pUL138\textsubscript{myc} and pUL133\textsubscript{HA}. Row 2) pUL138\textsubscript{(1-142)myc} + pUL133\textsubscript{HA}, lysate containing pUL138\textsubscript{(1-142)myc} and pUL133\textsubscript{HA}. Row 3) pUL138\textsubscript{(1-114)myc} + pUL133\textsubscript{HA}, lysate containing pUL138\textsubscript{(1-114)myc} and pUL133\textsubscript{HA}. Row 4) pUL138\textsubscript{(1-85)myc} + pUL133\textsubscript{HA}, lysate containing pUL138\textsubscript{(1-85)myc} and pUL133\textsubscript{HA}. Row 5) pUL138\textsubscript{(1-55)myc} + pUL133\textsubscript{HA}, lysate containing pUL138\textsubscript{(1-55)myc} and pUL133\textsubscript{HA}.

The pUL133 transmembrane domain is not involved in the interaction with pUL138

In addition to mapping the domains of pUL138 required for the interaction with pUL133 we hypothesized that the transmembrane domains of the CLAMPs might mediate the interactions between pUL133 and pUL138. Since all of the
CLAMPS localize to the Golgi and since, pUL135 and pUL138 do not interact, we tested this hypothesis by generating chimeric pUL133 and pUL135 constructs containing mismatched transmembrane domains. The transmembrane domain of pUL133 was replaced with the transmembrane domain of pUL135 generating pUL133(UL135TM)\textsubscript{HA}. Similarly, we generated pUL135(UL133TM)\textsubscript{HA} by replacing the transmembrane domain of pUL135 with the transmembrane domain of pUL133. If the pUL133 transmembrane domain regulated the interaction of pUL133 with pUL138, we would expect the chimeric pUL133(UL135TM)\textsubscript{HA} which lacks the pUL133 transmembrane, domain to no longer interact with pUL138. Conversely, while wild type pUL135 does not interact with pUL138, if the pUL133 transmembrane domain mediated the interaction between pUL133 and pUL138 we would then expect the chimeric pUL135(UL133TM)\textsubscript{HA} which now contains the pUL133 transmembrane domain, to interact with pUL138.

To test these hypotheses we transiently transfected wild type pUL133\textsubscript{HA} or pUL135\textsubscript{HA} with pUL138\textsubscript{myc} or each of the chimeric proteins with pUL138\textsubscript{myc} and analyzed the interactions by co-immunoprecipitation followed by immunoblot. As demonstrated previously (Fig. 16C, 18, and 22), immunoprecipitations from lysates containing pUL133\textsubscript{HA} and wild type pUL138\textsubscript{myc} indicate that pUL133 and pUL138 interact (Fig. 23, A, lane 3). While immunoprecipitation using the rabbit antibody to the myc epitope tag clearly precipitated pUL138\textsubscript{myc}, there was weak co-precipitation of pUL133\textsubscript{HA} (Fig. 23, A, lane 2). However, the
immunoprecipitation using the rabbit antibody to the HA epitope tag clearly
immunoprecipitated pUL133\textsubscript{HA} and co-precipitated pUL138\textsubscript{myc} (Fig. 23, A, lane 3)
while a no-antibody control immunoprecipitation failed to precipitate either protein
thereby indicating that the immunoprecipitations were specific (Fig. 23, A, lane 4).
Immunoprecipitations from lysates containing pUL138\textsubscript{myc} and the
pUL133(UL135TM)\textsubscript{HA} chimera using the rabbit antibody to the myc epitope tag
immunoprecipitated pUL138\textsubscript{myc} but failed to precipitate pUL133(UL135TM)\textsubscript{HA}
(Fig. 23, A, lane 6). Interestingly, immunoprecipitations using the rabbit anti-HA
antibody immunoprecipitated a small amount of pUL133(UL135TM)\textsubscript{HA} but also
co-precipitated a small amount of pUL138\textsubscript{myc} (Fig. 23, A, lane 7). Considering
the inefficient immunoprecipitation of the pUL133(UL135TM)\textsubscript{HA} construct, these
data suggest that the pUL133 transmembrane domain is not involved in the
interaction with pUL138.
Figure 23 The pUL133 transmembrane domain is not required for interaction with pUL138. HEK293T cells were transiently transfected with (A) pUL138\textit{myc} in combination with wild type pUL133\textit{HA}, or pUL133(UL135TM)\textit{HA}. (B) pUL138\textit{myc} in combination with wild type pUL135\textit{HA}, or pUL135(UL133TM)\textit{HA}, and screened for protein-protein interactions by immunoprecipitation followed by immunoblot. Immunoprecipitations utilized rabbit antibodies to the myc and HA epitope tags while immunoblots utilized mouse antibodies to the myc and HA epitope tags.

Further, immunoprecipitation from lysates containing pUL135\textit{HA} and pUL138\textit{myc} using the rabbit antibody to the myc epitope tag precipitated pUL138\textit{myc} but failed to co-precipitate pUL135\textit{HA} (Fig. 23, B, lane 2) as shown previously (Fig. 16D). Immunoprecipitation in the opposite direction using the rabbit antibody to the HA epitope tag, precipitated a small amount of pUL135\textit{HA} and co-precipitated a slight amount of pUL138\textit{myc} (Fig. 23, B, lane 3) just above the background precipitated by a no antibody control immunoprecipitation (Fig. 23, B, lane 4). Immunoprecipitation from lysates containing the pUL135(UL133TM)\textit{HA} chimera and pUL138\textit{myc} using the rabbit antibody to the HA tag not only precipitated pUL135(UL133TM)\textit{HA} but also co-precipitated a small amount of pUL138\textit{myc} (Fig. 23, B, lane 7). However, because the same
immunoprecipitation from lysates containing pUL135$_{HA}$ and pUL138$_{myc}$ also co-
precipitated a small amount of pUL138$_{myc}$, it is likely that with the increased
immunoprecipitation of pUL135(UL133TM)$_{HA}$ over wild type pUL135$_{HA}$, that this
was simply a nonspecific immunoprecipitation, especially considering the lack of
cooprecipitation in the opposite direction using the myc antibody (Fig. 23, B, lane 6). Further, immunoprecipitations using transmembrane deficient pUL133 and
pUL138 epitope tagged constructs failed to precipitate interacting proteins (data
not shown). While we cannot rule out the requirement of the pUL133 transmembrane domain in the interaction of pUL133 with pUL138, these
preliminary results suggest that the transmembrane domain of pUL133 is not
required for the interaction with pUL138.

**Discussion**

We have previously shown that pUL138 was required, but insufficient for,
HCMV latency in vitro, suggesting that other viral and cellular factors likely contribute to the latent infection (111). We hypothesized that pUL138 must interact with other viral proteins to mediate the latent infection. Our laboratory has subsequently identified 3 additional novel proteins pUL133, pUL135, and
pUL136, encoded on the UL138 transcripts (55). With all 4 of these novel ULb’
proteins encoded on the same transcripts, we surmised that these proteins might cooperate to promote the latent infection. To explore this exciting possibility, we
began by characterizing these novel UL138-related proteins (CLAMPs) (168) and
subsequently worked to identify potential interactions between these them (this work) in an effort to identify key viral and cellular components involved in the regulation of the latent HCMV infection.

To explore the possibility that the CLAMPs might interact, we began by examining the subcellular localization of each of these proteins during productive infection in fibroblasts. Previous work has shown that pUL138 localizes to the Golgi during productive infection (111). We demonstrated that like pUL138, pUL136 also predominantly localizes in the Golgi at 24 hours post infection and maintains its Golgi localization at 48 hours post infection in MRC5 fibroblasts (Fig. 14). We also, demonstrated that both pUL133 and pUL135 localize to the cell membrane at early times post infection (24 hours post infection) but by 48 hours post infection, these proteins also localize predominantly in the Golgi (Fig. 14). Further, when all 4 CLAMPS are exogenously expressed during productive infection with a recombinant HCMV strain lacking the entire CLAMP locus, these proteins co-localize at the a perinuclear compartment resembling the Golgi (Fig. 15). Taken together, these results suggested that the CLAMPs might work together at the Golgi to regulate the outcome of infection through an as yet unidentified mechanism.
Table 5 CLAMP Interactions.

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In addition to their Golgi co-localization, we have shown that several CLAMPs have the ability to interact when expressed transiently in mammalian cells (Table 5). Through a systematic one-on-one immunoprecipitation analysis in transiently transfected cells, we identified interactions between pUL133 and pUL138 (Fig. 16C) and between pUL136 and pUL138 (Fig. 16F). We also demonstrated weak interactions between pUL135 and pUL138 (Fig. 16D) and between pUL135 and pUL136 (Fig. 16E) suggesting the possibility that a complex of CLAMPs may function in HCMV infected cells. Furthermore, we have shown that each of the CLAMPs has the ability to self-associate (Fig. 17) suggesting that a potential CLAMP complex may exist involving not only interactions between the different CLAMPs, but also interactions of individual CLAMPs with themselves.

To better understand the interaction between pUL133 and pUL138, we mapped the interacting domains of pUL138 that were involved in the interaction with pUL133. Using several pUL138 C-terminal truncations, we mapped the pUL138 domain required for interaction with pUL133 to amino acids 85-115 in the cytosolic tail of pUL138 (Fig. 22). Further, we determined that the pUL133
transmembrane domain was not required for the interaction of pUL133 with pUL138 (Fig. 23) suggesting that domains in the cytosolic tails of pUL133 and pUL138 likely mediate the interaction of these two CLAMPs. Future studies will need to determine the importance of these novel interactions to the outcome of HCMV infection in different cell types including the latency permissive CD34+ HPCs. Recombinant HCMV strains with mutations in the key interacting domains of pUL133 and pUL138 will be screened in our in vitro latency model to identify interactions between these two proteins that are involved in modulating the outcome of infection. These novel interactions promise to reveal key secrets in the regulation of HCMV infection in different cell types and will likely identify key cellular pathways that contribute to HCMV latency and pathogenesis.
CHAPTER 4: pUL138 INTERACTS WITH THE CELLULAR HSP40 VARIANT
MRJ

Introduction

We have demonstrated a requirement for the pUL138 protein during a latent-like infection using an experimental latency model in primary CD34+ human hematopoietic progenitor cells (HPCs) (53, 111). pUL138 is encoded within the ULb' region of the genome that is unique to clinical strains of the virus, and as such, is dispensable for replication in cultured fibroblasts. The genes encoded within the ULb' region are likely important for virus dissemination, latency and pathogenesis in the host (30, 42, 100, 177). pUL138 is a 21-kDa protein that is expressed during both productive and latent infections. In cultured fibroblasts, pUL138 is expressed early in infection, but accumulates throughout infection. In CD34+ HPCs, pUL138 expression endures in the absence of expression of the 72-kDa and 86-kDa immediate early proteins (111, 168). The protein is anchored in Golgi membranes with the large C-terminal portion residing on the cytosolic face of the Golgi membranes (111). Strikingly, recombinant viruses lacking UL138 coding sequences replicate with increased efficiency in HPCs, exhibiting a loss of latency phenotype (53, 111). The function of pUL138 in modulating the outcome of infection however remains unknown. The localization and orientation of pUL138 in Golgi membranes suggests that this protein may function in modulating vesicular and protein trafficking, stress, or apoptosis.
While pUL138 is required for latency in CD34\(^+\) HPCs, it is not sufficient and other cellular and viral factors are required (111). We have recently demonstrated the existence of 3 additional proteins, pUL133, pUL135, and pUL136, encoded on the polycistronic transcripts encoding pUL138 (55). Like pUL138, these proteins are integral membrane proteins that localize to Golgi and have large C-terminal cytosolic domains (111, 168). The CLAMP locus, as would be expected for ULb\(^+\) sequences, is dispensable for replication in fibroblasts. However, this locus suppresses replication in CD34\(^+\) HPCs, presumably to favor latency, and augments replication in primary human endothelial cells (168). We have also identified interactions between pUL138 and other CLAMPs that may modulate the outcome of infection (Chapter 3).

Cellular determinants that function in conjunction with viral infection to promote a latent infection have not been identified. We hypothesize that pUL138 will function, at least in part, through the interaction with other cellular proteins. To better understand how pUL138 contributes to the latent infection in CD34\(^+\) HPCs, we sought to identify and characterize important protein-protein interactions in human hematopoietic cells. Through a yeast two-hybrid screen of a human bone marrow cDNA library we identified the Hsp40 mRNA variant 2, MRJ (MRJ) as a potential pUL138-interacting partner. We confirmed this interaction by immunoprecipitation from mammalian cells expressing MRJ and pUL138. In experiments coupling immunoprecipitation and mass spectrometry (IP/MS), we also identified several hsp40 variants associating with pUL138.
Using several different portions of the pUL138 cytosolic domain in both co-transformation assays and yeast matings, we identified a key domain within pUL138 that was required for the interaction with MRJ in yeast. Further, by truncation analysis of the pUL138 cytosolic tail, we mapped the domain of pUL138 required for the interaction with MRJ in mammalian cells. Interestingly, through a focused mutagenesis of the cytosolic tail of pUL138 we were unable to identify any single stretch of amino acids in the cytosolic tail of pUL138 that was responsible for the interaction with MRJ. Taken together, these data suggest that a non-linear epitope may be required for the interaction of pUL138 with MRJ. Preliminary data suggests that during productive infection in MRC5 fibroblasts, HCMV up regulates MRJ messages as early as 24 hours post infection and subsequently modulates MRJ expression thereafter. Further, recombinant viruses that fail to make pUL138 show increased levels of MRJ message suggesting that pUL138 might work to down-regulate a cellular stress response induced upon viral infection to favor viral persistence. This work represents the first identified cellular interacting partner of a known latency factor required for HCMV latency in CD34+ cells infected in vitro, and posits that pUL138 and MRJ may cooperate to regulate the outcome of infection.
Materials and Methods

Yeast

A yeast two-hybrid screen was performed using the Matchmaker Gold Yeast Two-Hybrid System (Clonetech, Mountain View, CA) and the Mate & Plate Library–Human Bone Marrow (Clonetech) according to manufacturers instructions. Yeast dual transformation and yeast matings were performed according to established protocols (Clonetech Yeast Protocols Supplement). Yeast prey vectors containing interacting prey sequences were isolated from Y187 yeast and Mach1 (Invitrogen) E. coli transformed with each vector prior to sequencing the plasmids to identify interacting proteins.

Mass Spectrometry

6 roller bottles of confluent MRC5 cells were infected with TB40E-UL138Flag at an MOI of 3 for 48 hours. Cells were washed 3 times with PBS, scraped, and pelleted at 450 RCF for 15 min at 4°C. Supernatants were aspirated and cells were resuspended in PBS and pelleted 2 additional times for a total of 3 washes. The cell pellet was weighed and resuspended in 100 µL/g of cells in PBS + 20 mM Hepes-K pH 7.4 + 1.2% Polyvinylpyrrolidone-40 buffer. The cell slurry was then forced through a 23-gauge hole and flash frozen over liquid nitrogen. Cells were stored at -80°C until use. Immunoprecipitation mass spec was described previously (123) and performed at the Medical College of Wisconsin (Milwaukee, WI) in the laboratory of Dr. Scott Terhune. Briefly:
Control data sets from immunoprecipitations from untagged HCMV infected MRC5 fibroblasts were used to establish the background data set of nonspecific flag antibody precipitated proteins. The precipitated protein data set was generated by subtracting the background data set from the sample data set yielding a high stringency data set for analysis of interacting partners.

**Plasmids**

**Yeast Plasmids**

The Matchmaker Gold Yeast Two-Hybrid Bait vector pGBKT7 (Clonetech) was modified by the addition of an NdeI site upstream of the start codon using the Phusion Site-Directed Mutagenesis Kit (Finnzymes) according to the manufacturers instructions, yielding pGBKT7(NdeI). Six UL138 baits representing different fragments of the transmembrane deficient pUL138 were cloned into the EcoRI and SalI sites of pGBKT7(NdeI), yielding pGBKT7-UL138\((25-169)\), pGBKT7-UL138\((133-169)\), pGBKT7-UL138\((25-130)\), pGBKT7-UL138\((54-146)\), pGBKT7-UL138\((25-79)\), and pGBKT7-UL138\((79-133)\). All constructs were confirmed by digestion and sequencing.

**Mammalian Expression Plasmids**

The lentiviral expression vector pCIG2-IRES-EGFP and its derivatives pCIG2-UL133\(_{myc}\)-IRES-EGFP, pCIG2-UL135\(_{myc}\)-IRES-EGFP, and pCIG2-UL136\(_{myc}\)-IRES-EGFP used in this study were described previously (55).
Additionally, pCIG2-IRES-EGFP was modified to express TagRFP instead of eGFP by PCR amplifying TagRFP from pTagRFP-N (Evrogen) and cloning it into the BglII and SalI sites in place of eGFP yielding pCIG2-IRES-TagRFP. pCIG2-MRJ\textsubscript{HA}-IRES-TagRFP was generated by amplifying the MRJ cDNA from pCMV-XL5-MRJ (Origene) using a reverse primer with an HA epitope tag (YPYDVPDYA) and cloning it into the XhoI and BamHI sites of pCIG2-IRES-TagRFP. Wild type pUL138 with a C-terminal myc tag were described previously (Chapter 3). All constructs were confirmed by digestion and sequencing.

Five, 15 alanine domain substitutions in pUL138\textsubscript{myc} were generated through Phusion mutagenesis (Finnzymes) according to the manufacturer's instructions), using pCIG2-UL138\textsubscript{myc}-IRES-EGFP as template. Each 15 amino acid mutation included 5 mutated amino acids from the upstream mutation such that each mutant shared 5 mutated residues. Alanine mutants include; pCIG2-UL138\textsubscript{(87-101)myc}, pCIG2-UL138\textsubscript{(97-111)myc}, pCIG2-UL138\textsubscript{(107-121)myc}, pCIG2-UL138\textsubscript{(117-131)myc}, pCIG2-UL138\textsubscript{(127-142)myc}. Mutational primers are included in Table 5. All constructs were confirmed by digestion and sequencing.
### Table 6 Primers for Plasmids qRT-PCR, and Recombinant HCMV Strains.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Orientation</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Yeast Bait Primers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGBK7 (+Ndel)</td>
<td>FWD</td>
<td>3GCCGCCCATATGGAGGAGCAGAAGC</td>
</tr>
<tr>
<td>pGBK7-UL138 (25-169)</td>
<td>REV</td>
<td>3TCGCCCTATAGTGAGTCGTATTACAAATTGCC</td>
</tr>
<tr>
<td>pGBK7-UL138 (25-130)</td>
<td>FWD</td>
<td>9gggGAATTCTGCTATCTAGCTACCATTGCC</td>
</tr>
<tr>
<td>pGBK7-UL138 (25-79)</td>
<td>REV</td>
<td>9gggGTGACCCACGCTTCTCTGCTGATTACG</td>
</tr>
<tr>
<td>pGBK7-UL138 (133-169)</td>
<td>REV</td>
<td>9gggGTGACCCGATCATGCAGTGCCTGACC</td>
</tr>
<tr>
<td>pGBK7-UL138 (54-146)</td>
<td>FWD</td>
<td>9gggGAATTCTAACGGGAATACGAGCAGC</td>
</tr>
<tr>
<td>pGBK7-UL138 (25-79)</td>
<td>REV</td>
<td>9gggGTGACCTCTGCTGACTCCCACCGGTACG</td>
</tr>
<tr>
<td>pGBK7-UL138 (79-133)</td>
<td>REV</td>
<td>9gggGAATTCCGATACCGGCTTCTCCCAGAC</td>
</tr>
<tr>
<td>pGBK7-UL138 (79-133)</td>
<td>REV</td>
<td>9gggGTGACCGGGAAAGCAAGGGCTGTTCTGC</td>
</tr>
<tr>
<td><strong>pUL138 Alanine Domain Mutant Primers</strong></td>
<td></td>
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</tr>
<tr>
<td>pCIG2-UL138 (87-101)myc</td>
<td>FWD</td>
<td>GCAGCGGCTGCCCGCGGCCGCCGCTACTACGTCGTGCGGATAACAGCC</td>
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<tr>
<td>pCIG2-UL138 (87-111)myc</td>
<td>REV</td>
<td>3CCGCCGACCGGCGCCGGCCGGCGCCGCTTGGAGAAACG</td>
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<tr>
<td>pCIG2-UL138 (107-121)myc</td>
<td>FWD</td>
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<tr>
<td>pCIG2-UL138 (117-131)myc</td>
<td>FWD</td>
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<tr>
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<td>REV</td>
<td>3CCGCCGACCGGCGCCGGCCGCGGCTACTACGTCGTGCGGATAACAGCC</td>
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<tr>
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<td>FWD</td>
<td>GCAGCGGCGCCGGCGGGCCGCGGCTACTACGTCGTGCGGATAACAGCC</td>
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<tr>
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<td>REV</td>
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<td>Primers</td>
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<tr>
<td>MRJ Primers</td>
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<td>pCIG2-MRJHA-IRES-TagRFP</td>
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<tr>
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</tr>
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<td>TB40E-UL138-GalK</td>
<td>CCGCGACGCAGTACGCAACCTGATGCATATTATCACCGTACATTATCATCA</td>
<td>GTCAAAACGACATTACCGCGATCCGCTCCCCTCTTTTTCTTTTTCTCATTCACCTGGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCTCATTCACCTGTTGACAATGTATCATCGGCA</td>
</tr>
<tr>
<td>TB40E-UL138Flag</td>
<td>CCGCGACGCAGTACGCAACCTGATGCATATTATCACCGTACATTATCATCA</td>
<td>GTCAAAACGACATTACCGCGATCCGCTCCCCTCTTTTTCTTTTTCTCATTCACCTGGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCTCATTCACCTGTTGACAATGTATCATCGGCA</td>
</tr>
</tbody>
</table>

* FWD, forward; REV, reverse

3. 5' phosphorylation

4. Enzyme sites are underlined.

5. Extra nucleotides are in lowercase
Cells

Primary human embryonic lung fibroblasts (MRC5) and human embryonic kidney 293T cells (ATCC, Manassas, VA) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 100 U/mL penicillin, and 100 µg/mL streptomycin. HeLa cells (ATCC) were maintained in DMEM supplemented with 10% normal calf serum (NCS), and 100 U/mL penicillin, and 100 µg/mL streptomycin. All cells were maintained at 37°C with 5% CO₂.

Viruses

HCMV Strains

The recombinant clinical HCMV strain TB40E and its derivatives, TB40E-UL138STOP and TB40E-Δ3.6 were described previously (Chapter 1 and (168)). The recombinant HCMV strain UL138TB40E-UL138Flag expressing pUL138 with a C-terminal 3x-Flag epitope tag was generated by a previously described two-step, positive and negative selection recombination procedure in SW105 E. coli (178) (111) (Chapter 2 and 3) using primers in Table 5.

Lentiviruses

Lentiviruses expressing MRJHA and pUL138myc were generated as described in Chapter 3.
**Immunoprecipitations**

HEK-293T cells on 10cm dishes were transfected with Lipofectamine 2000 according to the manufacturer’s instructions. Briefly, cells were rinsed once with phosphate buffered saline (PBS) (136.8 mM NaCl, 9.6 mM Na$_2$HPO$_4$, 2.68 mM KCl, 1.47 mM KH$_2$PO$_4$) and incubated at 37$^\circ$C in 8 mL Optimem. 6.93 µg of each plasmid was mixed in 500 µL of Optimem (Invitrogen) and 14.85 µL of Lipofectamine 2000 was mixed in 500 µL Optimem. Mixtures were incubated at room temperature for 5 min and then combined and allowed to incubate at room temperature for 30 minutes before being added to the cells. Cell media was changed 6 hours post transfection and replaced with normal growth media. Cells were harvested by trypsinization 48 hours post transfection and pelleted at 450 RCF for 6 minutes at room temperature. Supernatants were aspirated and cell pellets resuspended in PBS + 20 mM iodoacetamide (IAA) and pelleted a second time at 450 RCF for 6 minutes at room temperature. Supernatants were aspirated and pellets flash frozen by dipping in liquid nitrogen and stored at -80$^\circ$C. One day prior to immunoprecipitation, antibodies were mixed with Protein G Plus Agarose (Pierce) and incubated overnight at 4$^\circ$C. Briefly, 20 µl of settled agarose was washed 3 times with IP wash buffer (PBS + 0.1% NP40 + 20mM IAA) and mixed with respective immunoprecipitation antibodies (see table) and incubated overnight at 4$^\circ$C. The next day, cell pellets were lysed in IP lysis buffer (PBS + 1% Igepal CA-630 (NP-40), 20 mM IAA, 0.2mM PMSF, 1X HALT (Pierce), 1X EDTA), for 1 hour at 4$^\circ$C vortexing every 10 minutes. Lysates were
then cleared of nuclear debris by centrifugation at 14000 RCF for 20 minutes at 4°C. Supernatants were then mixed with Zysorbin (Invitrogen) at a final concentration of 0.25% with shaking for 30 minutes at 4°C. The Zysorbin was then pelleted at 10000 RCF for 5 minutes at 4°C. Final protein concentrations were determined by BCA assay (Pierce) and 100 µg of each protein lysate was analyzed by immunoprecipitation.

**Immunoblotting**

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using NuPage (Invitrogen) gels and transferred to 0.45-µm polyvinylidene fluoride (Immobilon-FL, Millipore) membranes. Membranes were blocked in Tris-buffered saline (TBS) (25mM Tris [pH 8.0], 137 mM NaCl, 3 mM KCl, 1.5 mM MgCl2, pH 8.0) plus 5% nonfat dry milk and 2.5 mg/mL BSA for 1 hour at 4°C. Membranes were incubated in primary antibodies in TBS with 2.5 mg/mL and 0.05% Tween 20 (TBS-BT) overnight at 4°C. Membranes were then washed 3 times for 15 minutes each in TBS-BT at room temperature prior to incubation with either goat-mouse IgG (H+L) Dylight 680/800-conjugated or goat anti-rabbit IgG(H+L) DyLight 680/800-conjugated secondary antibodies (Pierce). Membranes were then washed 3 times for 15 minutes each in TBS-BT and imaged using the Odyssey infrared imaging system (Li-Cor).
Quantitative Reverse Transcriptase PCR

A linear MRJ DNA standard curve was generated by PCR amplifying the MRJ ORF from pCMV-XL5-MRJ and using the fragment to make a serial dilution from 100 million copies to 0.01 copies. Total RNA was harvested using the Nucleospin RNAII kit (Machery-Nagel) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of input RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer's instructions. Quantitative reverse transcription PCR (qRT-PCR) was performed using the LightCycler 480 Probes Master (Roche) according to the manufacturer’s instructions along with Universal Probe Library (UPL) (Roche) probes and primers specific for MRJ and the prevalidated UPL human beta-actin gene assay (Roche). qRT-PCR conditions were as follows; 95°C for 10 minutes and then 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds. Reactions were run on a LightCycler 480 (Roche), and analyzed with the LightCycler 480 software (version 1.5) using the second derivative max method.

Results

pUL138 interacts with MRJ by yeast two-hybrid screen

We have previously characterized pUL138 as being functionally required but not sufficient for latency during in vitro infection of CD34+ hematopoietic progenitor cells (HPCs) (111). It is likely that pUL138 interacts with cell-specific factors to mediate the latent infection. To understand how pUL138 promotes
HCMV latency, we sought to identify cellular proteins that interact with pUL138 in cells of the myeloid lineage. We began our search for interacting cellular proteins by performing a yeast two-hybrid screen using a human bone marrow cDNA library in Y187 yeast and a pUL138 bait consisting of the entire pUL138 C-terminal portion (amino acids 25-169), excluding the N-terminus through the transmembrane domain (Fig. 24A). Following mating of this construct with the human bone marrow cDNA library, we screened 1.0625 X10E7 diploids with a mating efficiency of nearly 42%. Two putative interacting proteins were identified, DNAJB6 and ZNF262. The Hsp40 variant DNAJB6, herein referred to as MRJ, was of particular interest.
C

<table>
<thead>
<tr>
<th>Sample</th>
<th>DDOX</th>
<th>DDOXA</th>
<th>QDOXA</th>
</tr>
</thead>
<tbody>
<tr>
<td>P53 + Large T</td>
<td><img src="DDOX_P53.png" alt="Image" /></td>
<td><img src="DDOXA_P53.png" alt="Image" /></td>
<td><img src="QDOXA_P53.png" alt="Image" /></td>
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<tr>
<td>Lamin + Large T</td>
<td><img src="DDOX_Lamin.png" alt="Image" /></td>
<td><img src="DDOXA_Lamin.png" alt="Image" /></td>
<td><img src="QDOXA_Lamin.png" alt="Image" /></td>
</tr>
<tr>
<td>pUL138(25-139) + MRJ</td>
<td><img src="DDOX_pUL138.png" alt="Image" /></td>
<td><img src="DDOXA_pUL138.png" alt="Image" /></td>
<td><img src="QDOXA_pUL138.png" alt="Image" /></td>
</tr>
<tr>
<td>pUL138(133-169) + MRJ</td>
<td><img src="DDOX_pUL138.png" alt="Image" /></td>
<td><img src="DDOXA_pUL138.png" alt="Image" /></td>
<td><img src="QDOXA_pUL138.png" alt="Image" /></td>
</tr>
<tr>
<td>pUL138(25-130) + MRJ</td>
<td><img src="DDOX_pUL138.png" alt="Image" /></td>
<td><img src="DDOXA_pUL138.png" alt="Image" /></td>
<td><img src="QDOXA_pUL138.png" alt="Image" /></td>
</tr>
<tr>
<td>pUL138(64-146) + MRJ</td>
<td><img src="DDOX_pUL138.png" alt="Image" /></td>
<td><img src="DDOXA_pUL138.png" alt="Image" /></td>
<td><img src="QDOXA_pUL138.png" alt="Image" /></td>
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<tr>
<td>pUL138(25-79) + MRJ</td>
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<td><img src="QDOXA_pUL138.png" alt="Image" /></td>
</tr>
<tr>
<td>pUL138(79-133) + MRJ</td>
<td><img src="DDOX_pUL138.png" alt="Image" /></td>
<td><img src="DDOXA_pUL138.png" alt="Image" /></td>
<td><img src="QDOXA_pUL138.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Figure 24 Y2H Constructs, and Yeast Matting. 6 different portions of the pUL138 cytosolic domain were cloned into the yeast bait vector pGBK7 generating 6 unique myc-tagged pUL138 bait constructs. The relative strength of the detected interaction with MRJ is shown at right. Relative sizes are indicated by dashed lines and amino acid positions are shown. B) Y2H Gold Yeast were transformed with each of the 6 pUL138 baits and analyzed for pUL138 bait protein production by immunoblot using the mouse antibody to the myc epitope tag. C) Y2H Gold yeast containing each of the pUL138 baits were mated to Y187 yeast containing the identified prey vector expressing MRJ and diploids were plated on double drop out media containing the color indicator X-α-gal (DDOX), double drop out media containing X-α-gal and the antifungal aureobasidin A (DDOXA), or quadruple drop out media containing X-α-gal and aureobasidin A (QDOXA). The positive control mating (p53 and Large T) and the negative control mating (Lamin and Large T) are shown for reference.

To roughly map the domains of pUL138 required for the interaction with MRJ, we designed 5 additional pUL138 baits consisting of different portions of the pUL138 cytosolic domain (Fig. 24A). In order to test for pUL138 bait protein production, we transformed Y2H Gold yeast with each of these constructs and analyzed the lysates by immunoblot with a mouse antibody to the myc epitope tag. The yeast tolerated each of the pUL138 constructs and produced fusion proteins of the expected sizes (Fig. 24B). To roughly map the domains of pUL138 required for the interaction with MRJ, we co-transformed these 5 additional pUL138 fragments into Y2H Gold yeast along with pGADT7-MRJ, the isolated prey vector containing the partial MRJ mRNA. We then screened the co-transformants for interactions by plating the diploids on quadruple drop out media with the color indicator X-α-gal and the strong antifungal aureobasidin A (QDOXA) and (Table 6). Based on the color and number of the resulting colonies, the primary interaction domain consisted of the carboxy terminus
(aa133-146) (Table 6). While the co-transformation protocol suggested that carboxy terminal regions of the pUL138 cytosolic domain were important for the interaction with MRJ, this screening method was quite stringent as evidenced by the low number of colonies using the largest pUL138 construct (Table 6) and we did not expect to identify weak or transient interactions.

Table 7 Yeast Co-Transformation Screen.

<table>
<thead>
<tr>
<th>pGADT7-MRJ +</th>
<th># Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGBK7-UL138(25-169)</td>
<td>2</td>
</tr>
<tr>
<td>pGBK7-UL138(133-169)</td>
<td>23</td>
</tr>
<tr>
<td>pGBK7-UL138(25-130)</td>
<td>-</td>
</tr>
<tr>
<td>pGBK7-UL138(54-146)</td>
<td>12</td>
</tr>
<tr>
<td>pGBK7-UL138(25-79)</td>
<td>-</td>
</tr>
<tr>
<td>pGBK7-UL138(79-133)</td>
<td>-</td>
</tr>
</tbody>
</table>

In order to identify potentially weak or transient interactions, we performed yeast matings of Y187 yeast containing the MRJ prey vector with Y2H Gold yeast containing each of the pUL138 bait constructs. This screen of the pUL138:MRJ interaction by yeast mating confirmed the interactions observed in the co-transformation assays (Fig. 24C) and identified additional portions of pUL138 that interact with MRJ including the middle portions of pUL138, which also grew as blue colonies in the presence of aureobasidin A on stringent quadruple drop out selection media. Interestingly, the pUL138 bait containing only the middle portion of the pUL138 cytosolic domain (amino acids 79-133), failed to grow on quadruple drop out selective media with aureobasidin A (Fig. 24C) suggesting
that multiple portions of the pUL138 cytosolic domain may be required for interaction with MRJ. These studies suggested that specific portions of the pUL138 cytosolic tail are required for interaction with MRJ and that a nonlinear epitope may be involved in the interaction of these two proteins.

**pUL138 interacts with MRJ in mammalian cells**

We have previously shown that pUL138 is a Golgi-localized transmembrane protein and that the majority of the pUL138 C-terminus resides on the cytosolic face of Golgi membranes (111). Since our yeast two-hybrid screen utilized a transmembrane deficient portion of pUL138, we wanted to confirm this interaction using full-length pUL138. Therefore, we cloned pUL138 with a C-terminal myc epitope tag into the NheI and EcoRV sites of the mammalian expression vector pCIG2-IRES-EGFP generating an HCMV promoter driven pUL138\textsubscript{myc} construct, pCIG2-UL138\textsubscript{myc}-IRES-EGFP. Additionally, we cloned the MRJ ORF (Origene) with a C-terminal HA epitope tag into the XhoI and BamHI sites of pCIG2-IRES-TagRFP generating pCIG2-MRJ\textsubscript{HA}-IRES-TagRFP. We transiently transfected HEK293T cells with pCIG2-MRJ\textsubscript{HA}-IRES-TagRFP and pCIG2-138\textsubscript{myc}-IRES-EGFP and analyzed the cell lysates by co-immunoprecipitation using the rabbit anti-myc or rabbit anti-HA antibodies followed by immunoblot with the mouse anti-myc or mouse anti-HA antibodies, respectively.
Figure 25 pUL138 interacts with MRJ in mammalian cells. HEK293T cells were transiently transfected with pUL138\textsubscript{myc} and MRJ\textsubscript{HA} alone or in combination and screened for protein-protein interactions under various conditions by co-immunoprecipitation using rabbit antibodies to the myc and HA epitope tags followed by immunoblot with mouse antibodies to the myc and HA epitope tags. Row 1) pUL138\textsubscript{myc} + MRJ\textsubscript{HA}, lysate containing both pUL138\textsubscript{myc} and MRJ\textsubscript{HA}. Row 2) +SDS, lysate containing both pUL138\textsubscript{myc} and MRJ\textsubscript{HA} were boiled in 1% SDS prior to immunoprecipitation. Row 3) Mixed Lysates, a lysate containing only MRJ\textsubscript{HA} combined with a lysate containing only pUL138\textsubscript{myc}. Row 4) MRJ\textsubscript{HA} alone, lysate containing only MRJ\textsubscript{HA}. Row 5) pUL138\textsubscript{myc} alone, lysate containing only pUL138\textsubscript{myc}.

In lysates containing pUL138\textsubscript{myc} and MRJ\textsubscript{HA}, co-immunoprecipitation with the rabbit anti-myc tag antibody not only precipitated pUL138\textsubscript{myc} (Fig. 25, row 1, left panel, lane 3), but also precipitated MRJ\textsubscript{HA} (Fig. 25, row 1, right panel, lane 3). Similarly, co-immunoprecipitation with the rabbit anti-HA antibody precipitated both MRJ\textsubscript{HA} (Fig. 25, row 1, right panel, lane 5) and pUL138\textsubscript{myc} (Fig. 25, row 1, left panel, lane 5). A no antibody immunoprecipitation control failed to precipitate either protein (Fig. 25, row 1, both panels, lane 7). These results
demonstrate a specific interaction between pUL138 and MRJ and confirm the interaction identified by yeast two-hybrid. In nearly all immunoprecipitations, the supernatants were cleared of protein (Fig. 25, row 1, both panels, lanes 2 and 4), but not in the case of the no antibody control (Fig. 25, row 1, both panels, lane 6).

Importantly, immunoprecipitations from lysates containing both MRJ_{HA} and pUL138_{myc} which were boiled in 1% SDS (Fig. 25, row 2) prior to co-immunoprecipitation precipitate only the target of each antibody and fail to precipitate the interacting partner. Additionally, immunoprecipitations from a mixture of two lysates, one containing only MRJ_{HA} combined with one containing only pUL138_{myc}, precipitated only the targets of each antibody and failed to precipitate interacting partners (Fig. 25, row 3). Finally, to demonstrate specificity, immunoprecipitation with the rabbit anti-myc antibody from lysates containing only MRJ_{HA} failed to precipitate MRJ_{HA} (Fig. 25, row 4, right panel, lane 3). Conversely, immunoprecipitations with the rabbit anti HA-tag antibody from lysates containing only pUL138_{myc} failed to precipitate pUL138_{myc} (Fig. 25, row 5, left panel, lane 5). As a control for the antibody used in immunoblotting, lysates containing each protein are run in lane 8 of both panels (Fig. 25).

While pUL138 and MRJ interact in HEK293T cells when expressed exogenously, we wanted to know if pUL138 and MRJ interact in cells relevant to HCMV infection. To answer this question we generated lentiviruses expressing MRJ_{HA} and pUL138_{myc}, transduced MRC5 fibroblasts and analyzed the lysates 48 hours post transduction by co-immunoprecipitation followed by immunoblot as
done previously in Figure 25 with the addition of a UL138 specific antiserum. In lysates containing pUL138\textsubscript{myc} and MRJ\textsubscript{HA}, co-immunoprecipitation with the rabbit antiserum to UL138 not only precipitated pUL138\textsubscript{myc} (Fig. 26, row 1, left panel, lane 3), but also precipitated a small amount of MRJ\textsubscript{HA} (Fig. 26, row 1, right panel, lane 3). Similarly, co-immunoprecipitation with the rabbit anti-HA antibody precipitated both MRJ\textsubscript{HA} (Fig. 26, row 1, right panel, lane 7) and a small amount of pUL138\textsubscript{myc} (Fig. 26, row 1, left panel, lane 7).

Interestingly, immunoprecipitations with the rabbit anti myc-tag antibody precipitated pUL138\textsubscript{myc} (Fig. 26, row 1, left panel, lane 5) but failed to co-precipitate MRJ\textsubscript{HA} (Fig. 26, row 1, right panel, lane 5), perhaps due to the extremely low level of expression of MRJ\textsubscript{HA} in these cells. A no antibody immunoprecipitation control failed to precipitate either protein (Fig. 26, row 1, both panels, lane 9). These results demonstrate a specific interaction between pUL138 and MRJ in cells relevant to HCMV infection, and confirm the interaction identified in HEK293T cells.
Figure 26. pUL138 interacts with MRJ in cells relevant to HCMV infection. MRC5 fibroblasts were transduced with pUL138<sub>myc</sub> and MRJ<sub>HA</sub> expressing lentiviruses alone or in combination and screened for protein-protein interactions under various conditions by co-immunoprecipitation using a UL138 specific antiserum, or rabbit antibodies to the myc and HA epitope tags followed by immunoblot with mouse antibodies to the myc and HA epitope tags. Row 1) pUL138<sub>myc</sub> + MRJ<sub>HA</sub>, lysate containing both pUL138<sub>myc</sub> and MRJ<sub>HA</sub>. Row 2) +SDS, lysate containing both pUL138<sub>myc</sub> and MRJ<sub>HA</sub> were boiled in 1% SDS prior to immunoprecipitation. Row 3) Mixed Lysates, a lysate containing only MRJ<sub>HA</sub> combined with a lysate containing only pUL138<sub>myc</sub>. Row 4) MRJ<sub>HA</sub> alone, lysate containing only MRJ<sub>HA</sub>. Row 5) pUL138<sub>myc</sub> alone, lysate containing only pUL138<sub>myc</sub>.

Importantly, immunoprecipitations from lysates containing both MRJ<sub>HA</sub> and pUL138<sub>myc</sub> which were boiled in 1% SDS (Fig. 26, row 2) prior to co-immunoprecipitation precipitate only the target of each antibody and fail to precipitate the interacting partner. Additionally, immunoprecipitations from a mixture of two lysates, one containing only MRJ<sub>HA</sub> combined with one containing only pUL138<sub>myc</sub>, precipitated only the targets of each antibody and failed to precipitate interacting partners (Fig. 26, row 3). Lastly, to demonstrate
specificity, immunoprecipitation with the rabbit anti-myc antibody or with the UL138 antiserum, from lysates containing only MRJ_HA failed to precipitate MRJ_HA (Fig. 26, row 4, right panel, lanes 3 and 5). Conversely, immunoprecipitations with the rabbit anti HA-tag antibody or from lysates containing only pUL138_myc failed to precipitate pUL138_myc (Fig. 26, row 5, left panel, lane 7). Taken together, these results indicate that pUL138 and MRJ interact not only in mammalian cells lines but also in primary cells relevant to HCMV infection.

**pUL138 interacts with Hsp40 during productive infection**

We next wanted to know if pUL138 interacted with MRJ during productive infection. To answer this question, MRC5 fibroblasts were infected with a recombinant virus expressing a 3X-Flag epitope-tagged pUL138, TB40E-UL138_Flag (168) at an MOI of 3, and analyzed by immunoprecipitation/mass spectrometry (IP/MS). 48 hours post-infection, cells were harvested and processed for immunoprecipitation. Among other proteins, mass spectrometry identified several Hsp40 variants including DNAJ-B2, DNAJ-B12, DNAJ-B11, DNAJ-C13, DNAJ-A1, and DNAJ-A2 (Table 7). Our failure to detect an interaction between pUL138 and the MRJ variant of Hsp40 in fibroblasts may reflect cell type-specific expression of Hsp40 variants. The expression of the MRJ variant of Hsp40 has been shown to be differentially regulated in neurons (34). It is possible that, compared to hematopoietic cells (the cell source of the yeast cDNA library; MRC5 fibroblasts may express lower levels of MRJ
compared to other Hsp40 variants. Indeed, using a commercially available MRJ antibody, we were unable to detect endogenous MRJ in MRC5 fibroblasts (data not shown). Further, lentiviral delivery of a HA-tagged MRJ expression vector in MRC5 fibroblasts results in only modest levels of MRJ protein expression compared to the same vector expressing other irrelevant proteins (Fig. 26 and data not shown). Notably, other members of the heat shock protein family of chaperones including Hsp70 or Hsp90 were not co-precipitated with pUL138, demonstrating specificity of the interaction with Hsp40 variants. Taken together, these data suggest that pUL138 and Hsp40 variants specifically interact in mammalian cells, but that the Hsp40 variants that interact may be dependent on the cell type.

Table 8 IP/MS Cellular Interacting Proteins.

<table>
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<tr>
<th>Name</th>
<th>Description</th>
<th>Protein Prob.</th>
<th>Peptide Count</th>
<th>Scan Count</th>
<th>Percent Coverage</th>
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<td>ATP-binding cassette sub-family D member 3 (70 kDa peroxisomalmembrane protein) (PMP70).</td>
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**Distinct domains of pUL138 are required for interaction with MRJ**

To map the domains of pUL138 required for the interaction with MRJ, we analyzed the interaction potential of each pUL138 truncation by immunoprecipitation followed by immunoblot to detect each interacting partner. HEK293T cells were transiently transfected with each of the pUL138 C-terminal truncations in combination with our construct expressing MRJ<sub>HA</sub>. Cell lysates were analyzed 48 hours post transfection by immunoprecipitation using either the rabbit anti-myc antibody to pull down pUL138 or the rabbit anti-HA antibody to
pull down MRJ. Lysates were transferred to membranes and immunoblotted with the mouse anti-myc or the mouse anti-HA antibodies to detect interacting partners (Fig. 27). As demonstrated previously (Fig. 25), the full-length pUL138 interacted with MRJ and this complex was precipitated from supernatants in either direction using the antibodies to the myc or HA epitope tags (Fig. 27, row 1, both panels, lanes 3 and 5). In lysates containing the pUL138 truncation missing amino acids 143-169 (pUL138(1-142)myc) or 115-169 (pUL138(1-114)myc), immunoprecipitation with the rabbit anti-myc antibody co-precipitated MRJ\textsubscript{HA} (Fig. 27, rows 2 and 3, both panels, lane 3). Additionally, immunoprecipitation of MRJ\textsubscript{HA} with the rabbit anti-HA antibody from the same lysates precipitated pUL138(1-142)myc and pUL138(1-114)myc (Fig. 27, rows 2 and 3, both panels, lane 5). Further truncation of the pUL138 C-terminus including amino acids 86-169 (pUL138(1-85)myc) and amino acids 56-169 (pUL138(1-55)myc) resulted in complete loss of interaction with MRJ\textsubscript{HA}, as indicated by lack of co-immunoprecipitated products in either direction (Fig. 27, rows 4 and 5, both panels, lanes 3 and 5).
Figure 27 Specific domains of pUL138 interact with MRJ. HEK293T cells were transfected with MRJ<sub>HA</sub>, in combination with pUL138<sub>myc</sub>, pUL138<sub>(1-142)myc</sub>, pUL138<sub>(1-114)myc</sub>, pUL138<sub>(1-85)myc</sub>, or pUL138<sub>(1-55)myc</sub>, and screened for protein-protein interactions by co-immunoprecipitation followed by immunoblot. Immunoprecipitations utilized rabbit antibodies to the myc and HA epitope tags, while immunoblots utilized mouse antibodies to the myc and HA epitope tags. Row 1) pUL138<sub>myc</sub> + MRJ<sub>HA</sub>, lysate containing both pUL138<sub>myc</sub> and MRJ<sub>HA</sub>. Row 2) pUL138<sub>(1-142)myc</sub> + MRJ<sub>HA</sub>, lysate containing pUL138<sub>(1-142)myc</sub> and MRJ<sub>HA</sub>. Row 3) pUL138<sub>(1-114)myc</sub> + MRJ<sub>HA</sub>, lysate containing pUL138<sub>(1-114)myc</sub> and MRJ<sub>HA</sub>. Row 4) pUL138<sub>(1-85)myc</sub> + MRJ<sub>HA</sub>, lysate containing pUL138<sub>(1-85)myc</sub> and MRJ<sub>HA</sub>. Row 5) pUL138<sub>(1-55)myc</sub> + MRJ<sub>HA</sub>, lysate containing pUL138<sub>(1-55)myc</sub> and MRJ<sub>HA</sub>.

Together, these data suggest that a specific domain of pUL138 including amino acids 86-115 may be important for the interaction of pUL138 with MRJ in mammalian cells. Interestingly, the interacting domains identified in the yeast two-hybrid screen and in the immunoprecipitation analysis implicate different portions of the pUL138 cytosolic tail. These differences may be due to the use of large fusion protein constructs in the yeast screen as these fusion proteins may
attain an different final conformation and therefore may interact differently than the pUL138 sequence alone. Indeed, adding an N-terminal myc tag to pUL138 or adding TagRFP to the C-terminus of pUL138 resulted in unstable pUL138 constructs (data not shown). Further, the N-terminal transmembrane domain of pUL138 may be required for proper folding as adding TagRFP to the C-terminus of wild type pUL138 rendered the fusion protein unstable, but adding TagRFP to a transmembrane deficient pUL138 resulted in a stable pUL138-TagRFP fusion protein (data not shown).

In a preliminary effort to more specifically map the pUL138 domains responsible for the interaction with MRJ, we engineered 5 unique myc-tagged, pUL138 constructs with 15 amino acid domains substituted for alanines over the region of pUL138 from amino acids 86-142 (Fig. 28A). We then screened each of these constructs for the ability to interact with MRJ by co-immunoprecipitation analysis. HEK293T cells were transiently transfected with wild type pUL138_{myc} or each of the alanine domain mutants in combination with MRJ_{HA} and complexes were immunoprecipitated using the rabbit antibodies to the myc and HA epitope tags followed by immunoblot with mouse antibodies to the same epitope tags. As shown previously, pUL138 and MRJ interact (Fig. 25, 26, and 27) as immunoprecipitations in either direction co-precipitate the opposite protein (Fig. 28B, panel 1). Interestingly, none of the alanine domain mutants exhibited a loss of interaction with MRJ as immunoprecipitations of each mutant still precipitated a decent amount of MRJ (Fig. 28, all panels). Immunoprecipitations of MRJ in
each case, also precipitated substantial amounts of the unique pUL138 alanine
domain mutant (Fig. 28, all panels).

Together, these data implicate a potentially more complex interaction of
pUL138 with MRJ and suggest that a non-linear epitope of pUL138 may be
required for the interaction with MRJ. From the interaction data it is reasonable
to assume that each of the alanine block mutants at least partially co-localize
with pUL138 in the Golgi. Future studies will need to determine the subcellular
localization of each of these mutants for more complete understanding of the
importance of these regions of pUL138.
Figure 28 The pUL138:MRJ interaction may require a non-linear epitope of pUL138. A) Schematic of wild type pUL138 and 5 pUL138 mutants containing 15 amino acid alanine block substitutions. Each construct shares 5 mutated alanines with the construct immediately N-terminal to it. Each construct encodes a C-terminal myc tag. Substituted amino acid positions are indicated. B) HEK293T cells were transiently transfected with wild type pUL138\textsubscript{myc} or each of the alanine block mutants in combination with MRJ\textsubscript{HA} (B1-B6) and analyzed by immunoprecipitation followed by immunoblot. Immunoprecipitations utilized the rabbit antibodies to the myc and HA epitope tags. Immunoblots utilized mouse antibodies to the myc and HA epitope tags. No antibody control immunoprecipitations are included to demonstrate antibody specificity.

**Other CLAMPs interact with MRJ in mammalian cells**

Having roughly mapped the pUL138 domains required for the interaction of pUL138 with MRJ, we wanted to determine if any of the other CLAMP proteins encoded on the UL138 transcripts, including pUL133, pUL135 and pUL136, also interact with MRJ. To answer this question, HEK293T cells were co-transfected with constructs encoding MRJ\textsubscript{HA} and one of the CLAMP proteins, pUL133\textsubscript{myc}, pUL135\textsubscript{myc}, or pUL136\textsubscript{myc}. Cell lysates were analyzed 48 hours post transfection by immunoprecipitation followed by immunoblot. Protein complexes were precipitated using the rabbit anti-myc or rabbit anti-HA antibodies to precipitate the HCMV CLAMP protein or the MRJ protein, respectively. Immunoprecipitates were then immunoblotted with the mouse anti-myc or the mouse anti-HA antibodies to query for the reciprocal co-precipitation of viral proteins and MRJ, respectively.
Figure 29 The CLAMPs interact with MRJ in mammalian cells. HEK293T cells were transfected with MRJ\textsubscript{HA}, in combination with A) pUL133\textsubscript{myc}, B) pUL135\textsubscript{myc}, or C) pUL136\textsubscript{myc}, and screened for protein-protein interactions by co-immunoprecipitation followed by immunoblot. Immunoprecipitations used rabbit antibodies to the myc and HA epitope tags, while immunoblots used mouse antibodies to the myc and HA epitope tags. No antibody control immunoprecipitations are included for specificity.

Immunoprecipitations of MRJ\textsubscript{HA} from lysates containing both pUL133\textsubscript{myc} and MRJ\textsubscript{HA} using the rabbit antibody to the HA epitope tag co-precipitated pUL133\textsubscript{myc} (Fig. 29A). However, a reduced level of MRJ\textsubscript{HA} was co-precipitated in the reverse direction using the myc antibody to precipitate pUL133\textsubscript{myc} (Fig. 29A). These results indicate that pUL133 and MRJ interact, although the interaction is most apparent when the complex is precipitated using the HA tag to precipitate MRJ\textsubscript{HA}. By contrast, immunoprecipitation of pUL135\textsubscript{myc} or MRJ\textsubscript{HA} resulted in modest levels of the alternate protein (Fig. 29B). These results hint that pUL135 and MRJ interact weakly or transiently; further analysis will be required to determine the exact nature of this potential interaction. Immunoprecipitation of MRJ\textsubscript{HA} co-precipitated pUL136\textsubscript{myc} to levels equivalent to the protein present in
the lysates (Fig. 29C). In the reverse direction, MRJ$_{HA}$ was also robustly co-precipitated with pUL136$_{myc}$ (Fig. 29C). These results indicate an interaction between pUL136 and MRJ that reflects the interaction observed between MRJ and pUL138. Importantly, immunoprecipitations without antibodies fail to precipitate either the CLAMP or MRJ (Fig. 29A-C). Though it is tempting to speculate, our data support the possibility, but cannot definitively conclude an interaction between MRJ and pUL133 and pUL135. Nonetheless, clear interactions exist between MRJ and pUL136 and pUL138 that promise to provide novel insights into the function of MRJ in HCMV pathogenesis and persistence.

**MRJ is induced during productive HCMV infection**

Hsp40 represents one subfamily of the mammalian DNAJ chaperones involved in protein folding in response to cellular stress (64). The classic role of the Hsp40 co-chaperones is to stimulate the chaperone activity of Hsp70 and Hsp90 to refold misfolded proteins in response to cellular stress. MRJ is one of at least 41 putative Hsp40 variants identified by genome-wide screening of the human genome (95). In addition to the characteristic role as a co-chaperone, MRJ has been implicated in a diverse array of cellular processes including preventing Huntington aggregation (34), regulating cell cycle progression (185), modulating transcriptional repression in T cells (39), neuronal stem cell self-renewal (179), and organizing keratin filaments (71). With such a diverse array of cellular processes involving MRJ, it is likely that during HCMV infection, MRJ
participates in many of the cellular processes required for completion of the viral lifecycle and is likely an important modulator of the outcome of HCMV infection.

Having identified an interaction between MRJ and several of the CLAMPs, we wanted to know whether of not these novel interactions were physiologically relevant to HCMV infection. We have begun to try to answer this question by examining MRJ mRNA expression during productive HCMV infection. We mock-infected, or infected MRC5 fibroblasts with the clinical HCMV strain TB40E, the CLAMP locus deficient strain TB40E-Δ3.6, or the pUL138 deficient strain TB40E-UL138STOP and analyzed the expression of MRJ over the 72 hour time-course of infection, by quantitative, reverse transcriptase, real-time PCR. HCMV infection up regulated MRJ expression as TB40EWT shows an approximately 2.5 fold induction of MRJ over mock-infected cells throughout infection (Fig. 30, red bars). Further, by 24 hours post infection, cells infected with a virus lacking the entire CLAMP locus (TB40E-Δ3.6) showed a modest increase in MRJ expression over wild type infection, and subsequently MRJ levels returned to a level similar to that observed during wild type infection (Fig. 30, yellow bars). Interestingly, cells infected with a virus lacking only pUL138, show an approximately 7.5 fold increase in MRJ expression by 24 hours post infection, followed by a similar return to the level observed in wild type infection (Fig. 30). This data set is one representative of two preliminary experiments and needs to be repeated for validation of our findings. Intriguingly though, the pronounced induction of MRJ during infection with a pUL138-deficient virus suggests that pUL138 may act to
down-regulate a cellular stress response induced by viral infection. Further, a virus lacking all of the CLAMPs; pUL133, pUL135, pUL136, pUL138, showed a small induction of MRJ expression at 24 hours post infection, but importantly, the level of MRJ expression was only modestly above mock infected cells. Together, these data suggest that multiple viral factors within the CLAMP locus may work together with one or more cellular factors, potentially promoting a cellular stress response, while pUL138 works to counteract the stress response, thereby modulating the outcome of viral infection.

Figure 30 HCMV infection induces MRJ expression. MRC5 fibroblasts were mock infected, or infected with the recombinant HCMV strains, TB40E_{WT}, TB40E-Δ3.6, or TB40E-UL138STOP and MRJ mRNA expression was analyzed over the time-course from 0-72 hours post infection. RNA was isolated, reverse transcribed and analyzed by qRT-PCR relative to B-actin. Mock MRJ levels at each time point were set to 1.
Discussion

In an effort to understand the mechanism(s) by which pUL138 promotes the latent infection of HCMV in CD34+ HPCs we sought to identify important interacting cellular proteins by a yeast two hybrid screen of a human bone marrow cDNA library. Through this screen we identified the cellular heat shock protein 40 (Hsp40) transcript variant 2 (MRJ). Dual transformation and yeast mating confirmed the validity of this interaction in yeast and suggested that specific domains near the carboxy terminus of the pUL138 cytosolic tail mediate its interaction with MRJ (Fig. 24). Subsequent co-immunoprecipitation analysis in mammalian cells confirmed the pUL138:MRJ interaction (Fig. 25) and a C-terminal truncation analysis revealed that specific domains in the cytosolic tail of pUL138 are critical for its interaction with MRJ (Fig. 27). Importantly, fine mapping of the domain important for the pUL138:MRJ interaction failed to identify a single stretch of amino acids essential for maintaining the interaction, hinting that a non-linear epitope may be responsible for the pUL138:MRJ interaction (Fig. 28). Through immunoprecipitation coupled to mass spectrometry, we have shown that pUL138 interacts with several Hsp40 variants during productive infection in fibroblasts (Table 7). Additionally, our preliminary data has shown that the MRJ variant of Hsp40 is poorly expressed in MRC5 fibroblasts but it is induced upon infection by wild type HCMV (Fig. 30). Further, viruses lacking the CLAMPs show differential regulation of MRJ expression with the lack of pUL138 resulting in increased induction of MRJ early during infection (Fig. 30).
While the functional significance of the pUL138:MRJ interaction is currently unknown, it is likely that this interaction plays a role in the regulation of cellular stress resulting from viral infection. In addition to modulating cellular stress, heat shock proteins have been shown to be functionally important to the outcome of herpesvirus infection (10, 24, 90, 155, 160, 180). Importantly, our laboratory has recently shown that pUL138 is translated via an IRES that is stress inducible (55), suggesting that pUL138 may utilize its interaction with MRJ to promote viral latency in a cell-type specific manner as a viral persistence strategy during times of cellular stress. Taken together, these data implicate cellular chaperones as key mediators of viral replication and hint at their potential role in viral persistence. We hypothesize therefore, that pUL138 interacts with MRJ to modulate the cellular stress response to viral infection via an unknown mechanism that results in viral latency.
CHAPTER 5: DISCUSSION

Human cytomegalovirus (HCMV) coexists indefinitely in infected individuals through a poorly characterized latent infection in hematopoietic cells. We previously demonstrated a requirement for UL138 in promoting a latent infection in CD34+ hematopoietic progenitor cells (HPCs). UL138 is encoded on three co-terminal transcripts of, 1.7-, 2.7-, and 3.6-kilobases. We have shown that the UL138 protein product (pUL138) is necessary but insufficient for HCMV latency. The mechanisms by which pUL138 contributes to the latent infection are unknown, however other viral determinants are required for the latent infection. We identified 3 novel proteins pUL133, pUL135, and pUL136 encoded on the UL138 transcripts and have named this novel locus the CLAMP locus for cytomegalovirus latency associated membrane proteins. Similar to pUL138, pUL133, pUL135, and pUL136 are Golgi localized type I transmembrane proteins expressed with early kinetics during productive infection. We identified several interactions between these novel proteins and characterized an interaction between pUL133 and pUL138. Additionally, we have shown that each of these novel proteins has the ability to self-associate. A yeast two-hybrid screen identified an interaction between pUL138 and the heat shock protein 40 (Hsp40) variant MRJ. We confirmed this interaction in mammalian cells and mapped the pUL138 region involved in this interaction to a domain in the cytoplasmic tail of pUL138. We also demonstrated interactions of MRJ with pUL133 and pUL136. Importantly, pUL138 specifically interacts with Hsp40 variants during productive
infection. Preliminary data suggest that HCMV infection up regulates MRJ mRNA expression with recombinant viruses lacking pUL138 showing a disproportionate up regulation of MRJ. pUL138 is the first HCMV protein demonstrated to promote a latent infection. While the mechanisms by which pUL138 contributes to latency remain unknown, the interaction with other CLAMPs and with MRJ, suggest that pUL138 may cooperate with other CLAMPs to modulate the cellular stress response at the Golgi to promote HCMV latency.

While the productive HCMV infection has been well characterized, mechanisms contributing to HCMV persistence and latency remain poorly understood. Latent viral coexistence with the host requires the maintenance of viral genomes, suppression of viral replication, inhibition of antiviral responses (i.e., apoptosis, type I interferon response), and evasion the host immune response. Having established a latent infection, the virus retains the ability to reactivate from the latent state given an appropriate stimulus and, ultimately, replicate productively.

Based on the classic definition of viral latency, suppressing lytic viral gene expression and replication seems an obvious mechanism by which UL138 might promote HCMV latency. Consistent with this hypothesis, the latency-associated transcript (LAT) of HSV likely suppresses productive cycle gene expression by down regulating ICP0 expression (50, 169) as a microRNA. However, pUL138 did not directly down regulate expression from the MIEP or interact with IE proteins to suppress expression from the MIEP when expressed transiently (Fig.
10). Consistent with this finding, UL138-null viruses grow with similar kinetics as FIXwt in productively infected fibroblasts (Fig. 6C) (53). Our data do not exclude the possibility that pUL138 negatively regulates the immediate early promoter or proteins in a context-dependent manner. The effect of pUL138 on MIEP activity and IE1/IE2 protein expression and function has yet to be explored in the context of infection in HPCs.

UL138 transcripts and protein were detected in both productively infected fibroblasts and latently-infected HPCs (Fig. 5 and 7). While the expression of latency-associated genes during the lytic infection is not uncommon among herpesviruses (5, 148, 159), the role of UL138 during the productive infection, if any, is unknown. UL138 is dispensable for viral replication in fibroblasts (Fig. 6C). Similarly, the latency-associated transcripts (LAT) of HSV are expressed during both the lytic and latent infections, but a role for LATs in viral replication has not been described. However, LATs are differentially transcribed and spliced depending on the context of infection (32, 57). While pUL138 is synthesized in all infected cell types tested, the outcome of infection likely depends on the context-dependent balance of competing viral and cellular factors promoting virus replication or latency.

In fibroblasts, UL138 expression was dependent on IE gene expression (Fig. 4A). It is of interest then to speculate how UL138 is expressed in HPCs when IE genes are minimally transcribed (Fig. 5B) and no IE proteins are detected (Fig. 7C) even at very early times following infection. Several possible
scenarios exist to address the possibility that UL138 is expressed in the absence of IE gene expression: (i) pUL138 may be synthesized from RNAs packaged in virions or (ii) UL138 may be transcribed independently of IE expression in the context of HPC infection. Virion RNAs are packaged nonspecifically and relative to their abundance in the infected cell (165). Given the high levels of expression of UL138 transcripts in productively infected cells (Fig. 4A and 5A), it is likely that these transcripts are packaged and can perhaps serve as template for translation in newly infected cells. Indeed, we have detected UL138 transcripts among DNase-treated RNAs isolated from purified virions treated with RNAase prior to lysis. We have not determined if the UL138 transcripts present in virions are full length. However, we have detected pUL138 expression in fibroblasts infected with HCMV in the presence of actinomycin D, and inhibitor of transcription, (4 μg/ml) by either immunoblotting or immunofluorescence analyses (unpublished results, Whitaker and Goodrum) suggesting that at least some of the pUL138 may arise from these packaged transcripts. Overall though, these experiments suggest that pUL138 is likely translated de novo following infection of HPCs in the absence of IE gene expression. In concordance with this hypothesis, recent work in our laboratory has identified and characterized an internal ribosome entry site (IRES) within the UL136 ORF that is stress inducible and may therefore drive translation of pUL138 during infection in the absence of IE gene expression (55).

Depending on the context of infection, UL138 may be expressed independently of IE gene expression. During productive infection, herpesvirus
gene expression has historically been characterized as a temporally ordered cascade of IE, early, and late gene expression. This dogma is challenged by recent studies investigating CMV infection outside the context of cultured fibroblasts (33, 52, 54, 154). In these studies in myeloid cells, or HPCs viral gene expression was skewed towards genes that are not known to contribute directly to virus replication, was highly specific to the cell type or tissue, and did not reflect conventional viral kinetics. From these findings, we propose that the profile of viral gene expression is dictated by the unique milieu of the particular cellular environment. Accordingly, Epstein-Barr virus infection of B cells results in one of five possible profiles of viral gene expression depending on the differentiation and activation state of the cell (reviewed in (166)). Early interactions between the cell, virion components, and products of viral gene expression following viral entry likely dictate the resulting viral program of gene expression and ultimately the outcome of the infection.

The localization of pUL138 to the Golgi apparatus (Fig. 11) suggests that pUL138 contributes to latency through a novel mechanism not previously demonstrated for other herpesviruses. In addition to playing a critical role in intracellular protein trafficking and herpesvirus egress, the Golgi has been implicated in the regulation of apoptosis (23, 65) and the cellular stress response (65). While each of these processes represent candidate mechanisms by which pUL138 may function in viral latency, some are more plausible than others. Infected cell survival is essential to viral latency and is one mechanism by which
HSV LATs contribute to latency (13, 110). The localization of the anti-apoptotic m41 protein of murine CMV to the Golgi suggests a role for the Golgi in mediating apoptosis during viral infection (23). While pUL138 could contribute to latency by promoting cell survival we consider this possibility unlikely since neither fibroblasts nor CD34+ HPCs infected with UL138-null viruses exhibit decreased viability compared to cells infected with the wild type virus (data not shown).

HCMV proteins localized throughout the secretory pathway play important roles in viral egress and in modulating intracellular protein trafficking to suppress cell surface presentation of MHC-I (44, 96, 116). It is possible that in its central location in the secretory pathway, pUL138 contributes to latency by blocking late viral glycoprotein trafficking or viral egress (44). While we have no direct evidence to the contrary, transcripts encoding late structural proteins have not been detected during infection of CD34+ HPCs (33, 52, 54). If productive viral gene expression is suppressed in latently infected cells then it is unlikely that the virus requires a gene function to block the traffic of late proteins or the maturation of virions. Alternatively, pUL138 might act as a “molecular crossing guard” in the Golgi, regulating the movement and activity of viral and/or cellular proteins to orchestrate the switch from productive to latent infection.

Having previously shown that pUL138 was not sufficient for HCMV latency, and having shown that other viral and cellular factors likely contribute to the latent infection (111), we hypothesized that pUL138 interacted with other viral
proteins to mediate the latent infection of HCMV. We previously identified 3 additional novel proteins encoded on the large UL138 transcripts, pUL133, pUL135 and pUL136 (55). As all 4 of these novel ULb’ proteins are encoded on a set of common overlapping transcripts, we hypothesized that these proteins might work together to mediate the latent infection through some novel mechanism that may involve Golgi functions. To determine if these additional proteins cooperated with pUL138 in promoting the latent infection, our laboratory characterized these UL138-related proteins that were subsequently termed CLAMPs for HCMV latency associated membrane proteins (168). As part of the characterization we sought to identify potential interactions between these 4 proteins as well as to identify key interacting cellular components involved in the regulation of the latent infection.

We have shown previously that pUL138 localizes to the Golgi during productive infection (111) and we hypothesized that the novel CLAMPs might also localize in the Golgi perhaps to function with pUL138 to promote HCMV latency. Like pUL138, we demonstrated that pUL136 also predominantly localizes in the Golgi at 24 hours post infection and maintains its Golgi localization at 48 hours post infection in fibroblasts (Fig. 14) suggesting that pUL136 and pUL138 might interact at the Golgi to control the outcome of infection in latency competent cells to favor latency. Additionally, we demonstrated that pUL133 and pUL135 localize to the cell membrane at 24 hours post infection, but by 48 hours post infection, these proteins also localize
predominantly in the Golgi (Fig. 14). Further, preliminary data suggest, that when exogenously expressed together during HCMV infection with a recombinant HCMV strain lacking the entire CLAMP locus, all 4 of these proteins co-localize at least in part to a perinuclear structure resembling the Golgi (Fig. 15). These data suggest that the CLAMPs may work together at the Golgi to regulate the outcome of infection through an as yet unidentified mechanism.

In addition to demonstrating co-localization of the CLAMPs to the Golgi, we have shown that several CLAMPs have the ability to interact when expressed transiently in mammalian cells. Using a systematic immunoprecipitation analysis in transiently transfected cells, we identified interactions between pUL133 and pUL138 (Fig. 16C) and between pUL136 and pUL138 (Fig. 16F). Interestingly, we also identified several weak interactions between pUL135 and pUL138 (Fig. 16D) and between pUL135 and pUL136 (Fig. 16E) hinting that a potential CLAMP complex may exist in HCMV infected cells. In addition to interactions between the CLAMPs, we have also shown that each of the CLAMPs has the ability to self-associate (Fig. 17) suggesting that a potential CLAMP complex may exist that not only involves interactions between the different CLAMPs, but also involves interactions of individual CLAMPs with themselves.

To better understand the interaction between pUL133 and pUL138, we mapped the interacting domains of pUL138 that were involved in the interaction with pUL133. Using several pUL138 C-terminal truncations, we mapped the pUL138 domain required for interaction with pUL133 to amino acids 85-115 in
the cytosolic tail of pUL138 (Fig. 22). Further, we determined that the pUL133 transmembrane domain was not required for the interaction of pUL133 with pUL138 (Fig. 23) suggesting that domains in the cytosolic tails of pUL133 and pUL138 likely mediate the interaction of these two CLAMPs. While we do not yet know the significance of these interactions, they promise to reveal key secrets in the regulation of HCMV infection in different cell types and will likely identify key cellular pathways that contribute to HCMV persistence and pathogenesis.

Toward this end, we surmised that cell specific factors present only in cells permissive for latency (CD34+ HPCs) might interact with pUL138 to promote HCMV latency in. To address this question we performed a yeast two-hybrid screen of a human bone marrow cDNA library, which should be rich in cellular factors specific to hematopoietic cells. Through this screen we identified the cellular Hsp40 transcript variant 2, known as MRJ. Using both dual transformation and yeast mating, we confirmed the validity of this interaction in yeast and identified specific C-terminal domains in the cytosolic tail of pUL138, required for the interaction of pUL138 with MRJ (Fig. 24). We confirmed the pUL138:MRJ interaction by co-immunoprecipitation analyses in mammalian cells expressing both proteins transiently (Fig. 25). Further, through a C-terminal truncation analysis we identified domains in the cytosolic tail of pUL138 that were critical for its interaction with MRJ (Fig. 27) and these domains roughly match the regions identified in the yeast analyses. Interestingly, mutating the identified domains to alanine failed to identify a single stretch of amino acids essential for
maintaining the interaction (Fig. 28). These data suggested that not surprisingly, a non-linear epitope may be responsible for the pUL138:MRJ interaction. Further, through immunoprecipitation/mass spectrometry, we have shown that pUL138 interacts with several Hsp40 variants during productive infection in MRC5 fibroblasts (Table 7). While we were unable to detect an interaction with MRJ in these analyses, it is clear that pUL138 specifically interacts with Hsp40 as we failed to co-precipitate other Hsps. Additionally, our preliminary data has shown that the MRJ variant of Hsp40 is poorly expressed in MRC5 fibroblasts suggesting a tissue-specific expression of Hsp40 variants which could account for our inability to detect the pUL138:MRJ interaction during productive infection of primary fibroblasts. Somewhat surprisingly though, we detected a strong induction of MRJ mRNA during infection by wild type HCMV (Fig. 30) in fibroblasts. Furthermore, infection with viruses lacking the CLAMP locus exhibited differential regulation of MRJ expression and infection with pUL138 deficient viruses showed a marked induction of MRJ early during infection (Fig. 30) suggesting that pUL138 might regulate the cellular stress response early during productive infection.

Not surprisingly, herpesviruses have been shown to exploit the cellular heat shock response to benefit viral replication. Heat shock proteins have been implicated in numerous steps in the replication of alpha-herpesvirus genomes. During infection, HSV-1 compartmentalizes the various cellular chaperone pools to favor efficient viral replication as virus infection in cells devoid of heat shock
protein 27 (Hsp27) result in decreased viral replication (90) suggesting a complex system to regulate the functions of the host chaperones to allow viral DNA replication. Further, HSV-1 has been shown to directly utilize several heat shock proteins including the Hsp40 variant hTid-1 to facilitate the binding of the UL9 multimeric protein complex to the viral origin of replication (160). Additionally, HSV-1 requires Hsp90 for localization of its DNA polymerase to the nucleus in order for viral replication to take place (24) suggesting that HSV-1 uses various cellular heat shock proteins to promote viral genome replication. In addition to regulating alpha herpesviruses, cellular heat shock proteins have been implicated in the regulation of the KSHV and EBV latent programs (119, 155, 180). Intriguingly, cytomegalovirus infection induces several cellular chaperones during infection including Hsp70, GRP94 and the endoplasmic reticulum associated chaperone BIP to benefit viral infection (70, 132). Further, Hsp90 has also been implicated in the replication of HCMV as specific inhibitors of Hsp90 prevent HCMV replication in vitro (10). Importantly, our laboratory has recently shown that pUL138 is translated via an IRES that is stress inducible (55), suggesting that pUL138 may utilize its interaction with MRJ to promote viral latency during times of cellular stress in a cell-type specific manner perhaps as a viral persistence strategy. Taken together, these data implicate cellular chaperones as key mediators of viral replication and hint at their potential role in viral persistence.

We hypothesize therefore, that pUL138 contributes to HCMV latency by
modulating the cellular stress response to viral infection via an unknown mechanism. While the Golgi-apparatus is central to the function of the secretory pathway, the Golgi has also been suggested to act as a signal transducer for cellular stress (65). Golgi resident proteins have been linked to quality control and cellular stress signaling mechanisms in the secretory pathway (86, 120) and Golgi resident proteins have also been implicated in homeostatic mechanisms modulating ER stress (120). Additionally, the Golgi plays a central role in cell-cycle regulation (reviewed in (156)) with individual Golgi matrix proteins fulfilling a functional role as reorganizers of the Golgi during cell cycle progression (102), and modulating centrosome organization (75, 76).

Interestingly, productive HCMV infection has been shown to completely reorganize the structures of the secretory pathway to allow the virus to utilize a membrane rich cytoplasmic site immediately next to the nucleus to assemble virions (131). With such a drastic reorganization of the secretory compartment it is not surprising that upon infection HCMV induces a cellular stress response that the virus must then modulate in order to complete its lifecycle before the cell shuts down. It is interesting to consider the potential role of pUL138 in regulating Golgi stress induced by viral budding from Golgi membranes during the process of secondary envelopment. To date, HCMV has already been shown to modulate the ER-mediated unfolded protein response (UPR) to favor viral replication during productive infection (70). Strikingly, Isler et al 2005, report that HCMV induces the UPR early during productive infection, precisely when viral
glycoprotein synthesis is maximal, but that in spite of the induction, the virus regulates the different UPR signal transduction pathways to benefit viral replication. As in productive infection, it is likely that the regulation of HCMV latency is also mediated through a complex integration of cellular signals involved in the stress response, cellular differentiation and cell cycle regulation. The regulation of cellular stress in the establishment of a latent infection has not been explored but the ability of HCMV to commandeer the UPR machinery speaks to the importance of regulating cellular stress responses to control viral replication and survival. We hypothesize therefore, that UL138 contributes to HCMV latency by modulating the cellular stress response to viral infection to promote the latent infection.
Figure 31 Cellular stress, mTOR signaling and the innate immune response. mTOR transduces cellular stress and modulates the activity of the cellular translational machinery. mTOR links the cellular stress response to the innate immune response via IRF7. HCMV UL38 inhibits TSC1/2, activating mTOR and promoting both cap-dependent and UTR mediated translation. TSC; tuberous sclerosis complex, IRF7; interferon response factor 7, 4EBP1/2; Binding Protein, p70 S6K; P70 S6 Kinase, eIF4E; eukaryotic initiation factor 4E, rpS6; ribosomal protein S6. It is proposed that pUL138 feeds into this pathway at an as yet unidentified position.

Recently, HCMV UL38 has been shown to block the cellular response to stress and maintain protein translation in infected cells by abrogating the function of TSC2, a negative regulator of the mammalian target of rapamycin or mTOR (79, 97). By activating mTOR, HCMV maintains eukaryotic initiation factor 4E (eIF4E), and ribosomal protein S6 (rpS6) in an active state thereby allowing for both cap-dependent and UTR mediated translation. The maintenance of the
cellular translation machinery to promote viral protein synthesis during viral infection might allow HCMV to avert an otherwise detrimental antiviral stress response directed against the production of viral proteins. However, prior to viral protein synthesis, the cell mounts an immune response to viral infection. Upon HCMV entry, the type-I interferon response is the cell’s first line of defense against viral infection (129). Intriguingly, the link between cellular stress and the production of type-I interferons suggests that the initial cellular response to viral infection is also dependent on the ability of the cell to appropriately respond to stress (27, 35). Recent work indicates that inhibition of eIF4E and rpS6, the downstream effectors of mTOR, not only decrease protein synthesis but also result in decreased production of interferon–α (27, 35) and a potential inability to fight off virus infection. Additionally, in the presence of active mTOR, the 4E-BP proteins which normally inhibit eIF4E and prevent cap dependent translation are inactivated, leading to induction of cap dependent translation as well as increased interferon-α production. Type-I interferon production is also significantly enhanced in mouse embryonic fibroblasts (MEFs) deficient in the 4E-BP proteins 1 and 2 (35). Further, siRNA knockdown of the rpS6 activation proteins p70S6K1 and p70S6K2 result in decreased interferon-α (27). Taken together, these data suggest that the UPR, cellular stress response, and innate immune response are all intimately linked and contribute to the defense of infected cells. Previous studies characterized the ability of laboratory strains of HCMV to regulate the UPR but the ability of clinical HCMV strains to modulate
the cellular stress response has not yet been examined. HCMV might further modulate the cellular stress response and perhaps regulate the innate immune response through modulation of type-I interferon production. Furthermore, it is reasonable that HCMV modulates the stress response and the innate immune response in a cell type-dependent manner, such that a controlled stress response may be beneficial to the latent infection and not just productive infection, as shown previously (70).

This is the first report of identified interactions between pUL138 and both viral and cellular factors. The interactions of pUL138 with other CLAMPs and with MRJ suggest that pUL138 likely requires both cell specific factors as well as other viral factors to promote HCMC latency perhaps through modulation of the cellular stress response. In spite of these novel interactions however, the mechanism by which pUL138 promotes the latent infection remains unknown. To gain an understanding of the mechanisms of HCMV persistence, future studies will first need to confirm the CLAMP co-localization during both productive and latent infections in relevant cell types including CD34+ HPCs. While, we have preliminary direct immunofluorescent data (described in materials and methods Chapter 3) indicating that all 4 of the CLAMPs at least partially co-localize when expressed transiently during productive infection with a CLAMP locus deficient HCMV strain (Fig. 15), future work will need to confirm the CLAMP interactions during HCMV infection. It will be important to determine the relevance of these novel interactions to the outcome of infection, particularly in
relation to infection in different cell types that support either a productive (fibroblasts, or endothelial cells) or latent infection (CD34+ HPCs). Importantly, these studies will need to address not only the viral factors, but also the cellular components associated with specific cell types that likely contribute to HCMV persistence.

With these studies in mind, we have already generated a recombinant virus using the clinical HCMV strain TB40E (described in materials and methods, Chapter 3) that will allow us to answer these important questions. The recombinant virus, TB40E-Super, has each of the 4 CLAMPs engineered to express a unique C-terminal epitope tag in the same manner as was employed for the uniquely tagged expression constructs used in this study. With this virus we plan to perform co-localization studies during both productive and latent infections, as well as perform CLAMP interaction studies by immunoprecipitating each CLAMP and screening for CLAMP interactions during infection. Further, with each CLAMP uniquely tagged, this virus will allow us to perform immunoprecipitation coupled to mass spectrometry to identify cellular proteins that interact with each of the different CLAMPs during productive and latent infections. These studies will significantly increase the relevance of our previous work and will likely provide new insights into the function of these novel proteins. A thorough understanding of the mechanisms governing the establishment and maintenance of latent HCMV will provide new insights into HCMV pathogenesis.
and persistence and will likely aid in the development of novel treatments for HCMV infection and prevention of HCMV disease.


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