

An Endothelial Cell-Specific Requirement for the *UL133-UL138* Locus of Human Cytomegalovirus for Efficient Virus Maturation

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Human cytomegalovirus (HCMV) infects a variety of cell types in humans, resulting in a varied pathogenesis in the immunocompromised host. Endothelial cells (ECs) are considered an important target of HCMV infection that may contribute to viral pathogenesis. Although the viral determinants important for entry into ECs are well defined, the molecular determinants regulating postentry tropism in ECs are not known. We previously identified the *UL133-UL138* locus encoded within the clinical strain-specific *ULb'* region of the HCMV genome as important for the latent infection in CD34⁺ hematopoietic progenitor cells (HPCs). Interestingly, this locus, while dispensable for replication in fibroblasts, was required for efficient replication in ECs infected with the TB40E or fusion-inducing factor X (FIX) HCMV strains. ECs infected with a virus lacking the entire locus (*UL133-UL138*_{NULL} virus) complete the immediate-early and early phases of infection but are defective for infectious progeny virus production. ECs infected with *UL133-UL138*_{NULL} virus exhibited striking differences in the organization of intracellular membranes and in the assembly of mature virions relative to ECs infected with wild-type (WT) virus. In *UL133-UL138*_{NULL} virus-infected ECs, Golgi stacks were disrupted, and the viral assembly compartment characteristic of HCMV infection failed to form. Further, progeny virions in *UL133-UL138*_{NULL} virus-infected ECs inefficiently acquired the virion tegument and secondary envelope. These defects were specific to infection in ECs and not observed in fibroblasts infected with *UL133-UL138*_{NULL} virus, suggesting an EC-specific requirement for the *UL133-UL138* locus for late stages of replication. To our knowledge, the *UL133-UL138* locus represents the first cell-type-dependent, postentry tropism determinant required for viral maturation.

Cytomegaloviruses (CMV) are large, complex DNA viruses belonging to the *Herpesviridae* family. Human CMV (HCMV) is one of eight human herpesviruses and is ubiquitous among the population worldwide. HCMV entrenches a lifelong persistence in its host through both chronic and latent states of infection (1–3). Subclinical reactivation and low-level virus shedding events likely occur intermittently throughout the lifetime of the immunocompetent host (1). However, in individuals with compromised T cell immunity, including transplant patients, AIDS patients, and cancer patients undergoing intensive chemotherapy regimens, HCMV reactivation from latent to productive states of infection is a common cause of morbidity and mortality (4, 5). Further, HCMV persistence has been associated with increased risk of age-related pathologies including atherosclerosis, immune senescence, and frailty (6–11). The molecular mechanisms governing HCMV persistence in the infected host are largely unknown, in part, because the viral determinants and cellular reservoirs of persistence are ill defined.

HCMV infects a diverse array of cell types in humans (5, 12, 13). Among the cells infected, vascular endothelial cells (ECs) are thought to be important for viral pathogenesis in the host. By nature of the EC boundary formed between the circulation and organ tissue, ECs serve as an important interface for bidirectional virus spread (1, 14). While ECs are considered a key target of CMV infection in humans (12) and mice (15), ECs have different susceptibilities and support distinct modes of infection *in vitro* depending on their anatomical source (i.e., lung, dermis, or intestine) and type (micro- versus macrovascular) (16–20). This cell-type- or cell origin-dependent gradation in viral replication suggests that EC-specific determinants control infection outcomes ranging from lytic replication to “smoldering persistence” and possibly latency (17, 21). Infected ECs contribute to virus dissemination and pathogenesis, in part through the infection of circu-

lating leukocytes or polymorphonuclear cells (1, 22–26) and by promoting monocyte recruitment and extravasation, possibly increasing vascular permeability for dissemination of infected circulating cells into tissues (27). Further, infected ECs may detach and circulate (28, 29). These modes of cell-cell-based dissemination likely define the contribution of ECs to viral spread in the host (30). Based on studies using human, murine (MCMV), or rat CMV, infection of ECs is associated with persistent proinflammatory signaling contributing to angiogenesis and vascular diseases including atherosclerosis and atherothrombosis (7, 31–38). Accordingly, HCMV is a risk factor for restenosis following angioplasty and for transplant vascular sclerosis and chronic graft rejection following solid organ allograft transplantation (39–42). Understanding the biology of HCMV infection in ECs is critical given the pivotal role of ECs in HCMV infection and pathogenesis.

As EC-specific determinants impact HCMV replication, HCMV strains also exhibit a gradation in their ability to enter and replicate in ECs (43, 44). Well-characterized viral determinants of endothelial tropism predominantly function in viral entry into ECs. Proteins encoded by *UL128*, *UL130*, and *UL131A* complex with glycoproteins H (gH) and L (gL) on the virion surface to mediate entry into endothelial cells, leukocytes, and dendritic cells by endocytosis (23, 45–50). This complex is not required for entry into fibroblasts; rather, HCMV enters fibroblasts by fusion mediated by the gH/gL/gO complex (51–53). HCMV strains passaged

Received 14 September 2012 Accepted 22 December 2012

Published ahead of print 2 January 2013

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doi:10.1128/JVI.02510-12

TABLE 1 Primary antibodies used for immunofluorescence and immunoblotting

Antigen	Antibody	Type ^a	Source	Dilution used for:	
				Immunofluorescence ^{b,d}	Immunoblotting ^{c,d}
EEA-1	14/EEA1	M	BD Transduction	1:250	ND
UL44	10D8	M	Virusys	ND	1:2,500
Golgi marker proteins					
giantin	Custom	M	Gift ^e (Abcam)	1:250	ND
GM130	Clone 35	M	BD Transduction	1:100	ND
	EP892Y	R	Abcam	1:250	ND
GS27	25	M	BD Transduction	1:1,100	ND
β-Actin	ACTN05(C4)	R	Abcam	ND	1:1000
pp28	10B4-29	M	Gift ^f	1:20	1:50
IE1/IE2	3H4	M	Gift ^g	ND	1:100
gB	27-156	M	Gift ^h	1:30	ND
CD63	SC-5275	M	Santa Cruz	1:250	ND

^a R, rabbit; M, mouse monoclonal.

^b Dilution in PBS supplemented with bovine serum albumin and Tween 20.

^c Dilution in TBS-5% milk supplemented with bovine serum albumin and Tween 20.

^d ND, not done.

^e Generous gift from Samuel Campos, University of Arizona.

^f Generous gift from Michael Nevels, University of Regensburg.

^g Generous gift from Tom Shenk, Princeton University.

^h Generous gift from Bill Britt, University of Alabama at Birmingham.

in fibroblasts accumulate mutations in the *UL128-UL131A* locus, resulting in lost tropism for endothelial and epithelial cells (23, 47, 49, 50, 54) and reduced transfer of virus to leukocytes (23). However, laboratory strains competent for entry into ECs (e.g., encoding wild-type [WT] or repaired *UL131A*) still replicate to reduced yields relative to several clinical strains (23, 49). Further, various clinical isolates of HCMV replicate with different efficiencies in ECs (19, 44, 55, 56), suggesting the existence of viral determinants specific to clinical strains that control postentry EC tropism. Few CMV-coded determinants regulating postentry EC tropism have been defined. Studies in MCMV revealed a requirement for the M45 gene to inhibit apoptosis for productive replication specifically in ECs and macrophages (57). Recently, the *US16* HCMV gene was reported to be required for delivery of viral genomes and tegument proteins to the nuclei of ECs but not fibroblasts (58). Viruses containing disruptions in *US16* are defective at the immediate-early (IE) stage and all subsequent stages of infection in ECs. These studies support the notion that CMV has evolved functions to specifically overcome tropism barriers to infection in unique cell types and emphasize the importance of the cellular environment in ultimately determining the success of infection.

In the present study, we investigated the requirement for the *ULb'* region-encoded *UL133-UL138* locus for replication in primary human lung ECs. This locus encodes four proteins, pUL133, pUL135, pUL136, and pUL138, from polycistronic transcripts (59, 60). Like the entire *ULb'* region, the *UL133-UL138* locus is dispensable for viral replication in fibroblasts (60). However, the locus functions to suppress virus replication in CD34⁺ hematopoietic progenitor cells (HPCs), contributing to the latent phenotype *in vitro* (60, 61). Interestingly, this locus is required for efficient viral replication in multiple types of primary human ECs (60). The defect observed in ECs was independent of the multiplicity of infection (MOI) and the *UL138* latency determinant. Here, we characterize this defect and determine that the *UL133-*

UL138 locus is required for the late stages of infection and virus maturation specifically in ECs. ECs infected with a virus lacking the *UL133-UL138* locus (*UL133-UL138*_{NULL}) had dramatically altered organization of intracellular membranes and failed to form the viral assembly compartment characteristic of HCMV infection. These alterations in intracellular membranes correlated with a failure to produce mature progeny virions with tegument and secondary envelope. The dissimilarities between WT and *UL133-UL138*_{NULL} virus infections were unique to ECs. These studies indicate that one or more proteins encoded within the *UL133-UL138* locus contribute to the reorganization of intracellular membranes and, consequently, to secondary envelopment and egress in ECs. To our knowledge, these studies point to the first EC-specific viral determinants required for virus maturation.

MATERIALS AND METHODS

Cells. Human embryonic lung fibroblasts (MRC-5) (purchased from 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 microvascular lung ECs (HMVEC) (purchased from Lonza, Walkersville, MD) were cultured in EGM-2 MV BulletKit medium (Microvascular Endothelial Cell Growth Medium-2; Lonza) with 5% FBS. All cells were cultured at 37°C in 5% CO₂.

Viruses. Bacterial artificial chromosome (BAC) clones of the HCMV TB40E or fusion-inducing factor X (FIX) parental (WT) (13) strain and the *UL133-UL138*_{NULL} variant express the green fluorescent protein (GFP) as a marker of infection (60). The construction of the *UL133-UL138*_{NULL} variant was described previously (60, 61). Virus stocks were propagated by transfecting the viral BAC genome into MRC-5 fibroblasts and stored as described previously (61). Infectious virus yields were determined by 50% tissue culture infective dose (TCID₅₀) on MRC-5 fibroblasts. Centrifugal enhancement was used to increase the efficiency of FIX infection in ECs. For viral growth curves, virus yields were measured by TCID₅₀ from cell lysates harvested over a time course.

Virus entry. HMVEC were plated in 12-well dishes and infected the following day at an MOI of 0.2. The infection was enhanced by centrifuging plates at $250 \times g$ for 20 min immediately following infection. To remove unabsorbed virus, infection medium was replaced with fresh medium at 6 h postinfection (hpi). Cells were collected at 48 h postinfection and stained with propidium iodide. GFP was measured in live cells as an indicator of infection using an LSRII instrument (BD Biosciences, San Jose, CA).

Immunoblotting. Immunoblotting was performed as described previously (61). Briefly, 15 to 20 μ g of protein lysates was separated on 12% bis-Tris gels by electrophoresis and transferred to 0.45- μ m-pore-size polyvinylidene difluoride (Immobilon-FL; Millipore, MA) membranes. The proteins were immunoblotted using mouse monoclonal or rabbit polyclonal antibodies directed against each protein and detected using fluorescently conjugated secondary antibodies and the Odyssey infrared imaging system (Li-Cor, NE). All antibodies used are listed in Table 1.

Transmission electron microscopy. HMVEC or MRC-5 cells were mock infected or infected at an MOI of 4 or 2, respectively, with centrifugal enhancement. Infection medium was replaced at 24 h postinfection for HMVEC and at 6 h postinfection for MRC-5 cells with normal growth medium for each respective cell type. All cells were harvested at 5 days postinfection (dpi). Cells were fixed in 2.5% glutaraldehyde and 0.1 M PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] for 20 min. The fixed cell pellet was postfixed with osmium tetroxide in 0.1 M PIPES and dehydrated in a graded series of alcohol. Pellets were infiltrated with resin and cut into 100-nm sections. The sections were floated onto copper grids and imaged using a Phillips CM-12s transmission electron microscope. Cells were embedded and sectioned by the Arizona Research Laboratories, Arizona Health Sciences Center Core Facility.

Indirect immunofluorescence. Indirect immunofluorescence was performed as described previously (39). Briefly, HMVEC and MRC-5 cells were seeded onto 12-mm glass coverslips in 24-well dishes 1 day prior to infection at an MOI of 2. Cells were processed for indirect immunofluorescence at 144 hpi for HMVEC and at 96 hpi for MRC-5 cells. Cells were fixed in 2% formaldehyde in phosphate-buffered saline (PBS) and permeabilized. Primary and secondary antibodies (Table 1) were incubated in PBS-Tween 20 (PBS-T) for 1 h. Poststaining, DNA was stained with 4',6'-diamidino-2-phenylindole (DAPI) according to the manufacturer's instructions (Molecular Probes). Coverslips were mounted using Aqua Polymount according to the manufacturer's instructions (Polysciences, Inc.). Cells were imaged using a Zeiss 510 Meta Confocal Microscope as a lambda stack and unmixed using the Zeiss 510 Meta software, version 4.2, and images were processed using Zeiss software.

RESULTS

***UL133-UL138* is required for efficient replication postentry in endothelial cells.** We previously determined that the *ULb'*-coded *UL133-UL138* locus is required for efficient replication in primary human ECs such that a virus lacking this locus (*UL133-UL138*_{NULL} virus) exhibited a replication defect in a variety of ECs relative to wild-type (WT) virus (60). *UL133-UL138*_{NULL} virus infection results in a 5- to 1,000-fold defect in virus yields relative to the WT infection in five distinct primary EC types, including both microvascular and macrovascular cells. This defect is specific to ECs as the *UL133-UL138* locus is dispensable for replication in human fibroblasts and epithelial cells (60). To further explore this phenotype, primary human microvascular ECs (HMVEC) of the lung were infected at a low MOI (0.05 TCID₅₀ per cell), and virus yields were measured over a time course (Fig. 1A). *UL133-UL138*_{NULL} virus infection resulted in an overall 1,000-fold defect relative to the WT. A similar defect was observed in HMVEC infected with the FIX strain of HCMV (Fig. 1B). This replication defect, taken together with our previous work, indicates a strong

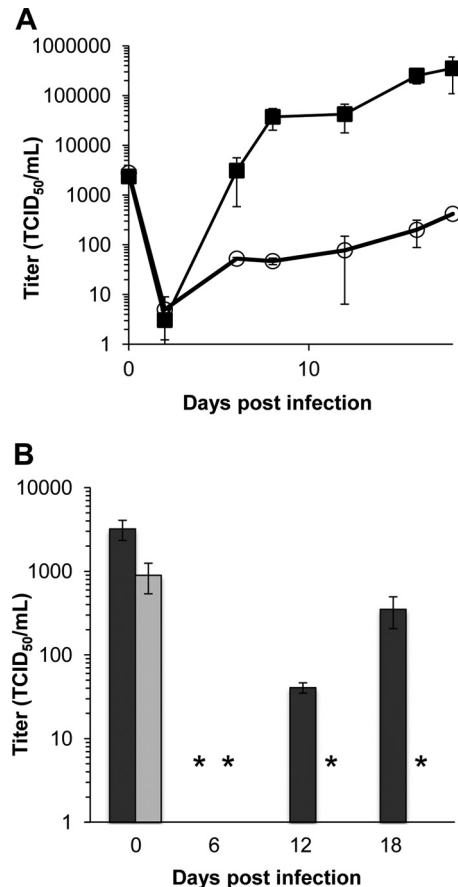


FIG 1 *UL133-UL138* is required for viral replication in ECs. HMVEC were infected with WT or *UL133-UL138*_{NULL} virus of either the TB40E (A) or FIX (B) strain at an MOI of 0.05, and virus yields were measured over a time course by TCID₅₀ on fibroblasts. WT is represented by the filled squares (A) or dark bars (B). *UL133-UL138*_{NULL} virus is represented by the open circles (A) or light bars (B). The values plotted are averages from three independent experiments, and the standard deviations are indicated by the error bars for each time point. In some cases, the error bars are too small to be seen. In panel B, the asterisks indicate values at the limit of detection.

dependence on the *UL133-UL138* locus in multiple endothelial cell types.

Most known restrictions to HCMV tropism in ECs occur early in the infection process, residing at the level of viral entry (62) or delivery of the viral genome to the nucleus (58). As such, one plausible explanation for the observed defect in replication may be that a protein encoded in the *UL133-UL138* locus is required for viral entry into ECs, similar to the *UL128-UL131A* locus. As both the WT and *UL133-UL138*_{NULL} viruses used in this work have been engineered to express the green fluorescent protein (GFP) as a marker for infection, the expression of GFP can be used as an indicator of both entry and delivery of the viral genome to the nucleus. We measured the proportion of GFP-positive (GFP⁺) HMVEC infected with either the WT (26.6%) or *UL133-UL138*_{NULL} (26.9%) virus at 48 hpi, a time preceding secondary rounds of infection (Fig. 2A). Analysis of the mean fluorescence intensities of the infected populations in four experiments indicates that the WT and *UL133-UL138*_{NULL} viruses enter, deliver the genome to the nucleus, and express GFP similarly in HMVEC (Fig. 2B). Based on this result, the EC-specific replication defect

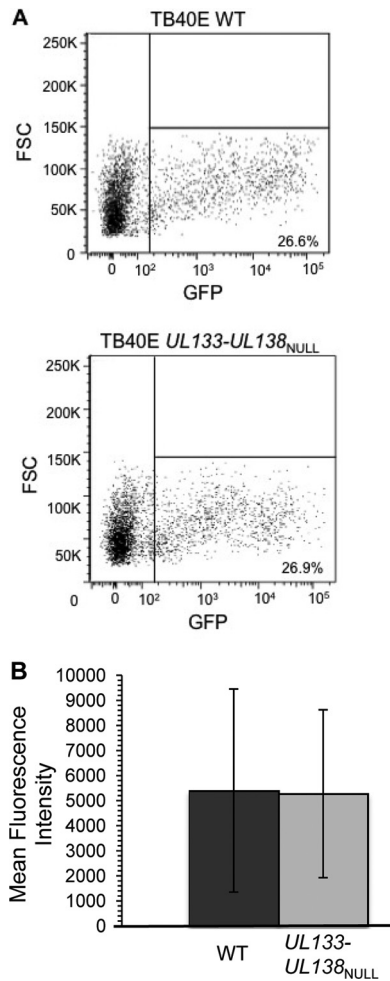


FIG 2 *UL133-UL138* is not required for virus entry or genome transit to the nucleus in ECs. HMVEC were infected with TB40E-WT or -*UL133-UL138*_{NULL} virus at an MOI of 0.02. Infected (GFP⁺) cells were analyzed at 48 hpi by flow cytometry. (A) Representative dot plots with the percentage of infected cells in each infection indicated. (B) The average mean fluorescence intensities for four independent experiments for each infection are shown, and the standard deviations are indicated by the error bars. FSC, forward scatter.

exhibited by the *UL133-UL138*_{NULL} virus cannot be attributed to a defect in viral entry or trafficking to the nucleus.

The *UL133-UL138* locus is required for maximal late viral gene expression. We next compared the accumulation of representative immediate-early (IE), early, and late proteins in HMVEC infected with TB40E-WT or -*UL133-UL138*_{NULL} virus to define the postentry point at which viral replication is constrained in ECs. HMVEC were infected at an MOI of 2 and the IE1 72-kDa and IE2 86-kDa proteins (IE proteins), pUL44 (early protein; viral DNA polymerase processivity factor), and pp28 (late protein; prominent constituent of the tegument) were analyzed over a time course by immunoblotting (Fig. 3). IE1 and pp28 bands on immunoblots from four to six experiments were quantitated to compare the differences in protein accumulation (Fig. 3B and C). IE1 (Fig. 3B) and UL44 (data not shown) accumulated to WT levels in *UL133-UL138*_{NULL} virus-infected HMVEC. pp28 levels were reduced 1.4- to 2-fold in the *UL133-UL138*_{NULL} virus infection relative to levels in WT infection. However, these differences were not statistically significant ($P \geq 0.1$) (Fig. 3C). These results indi-

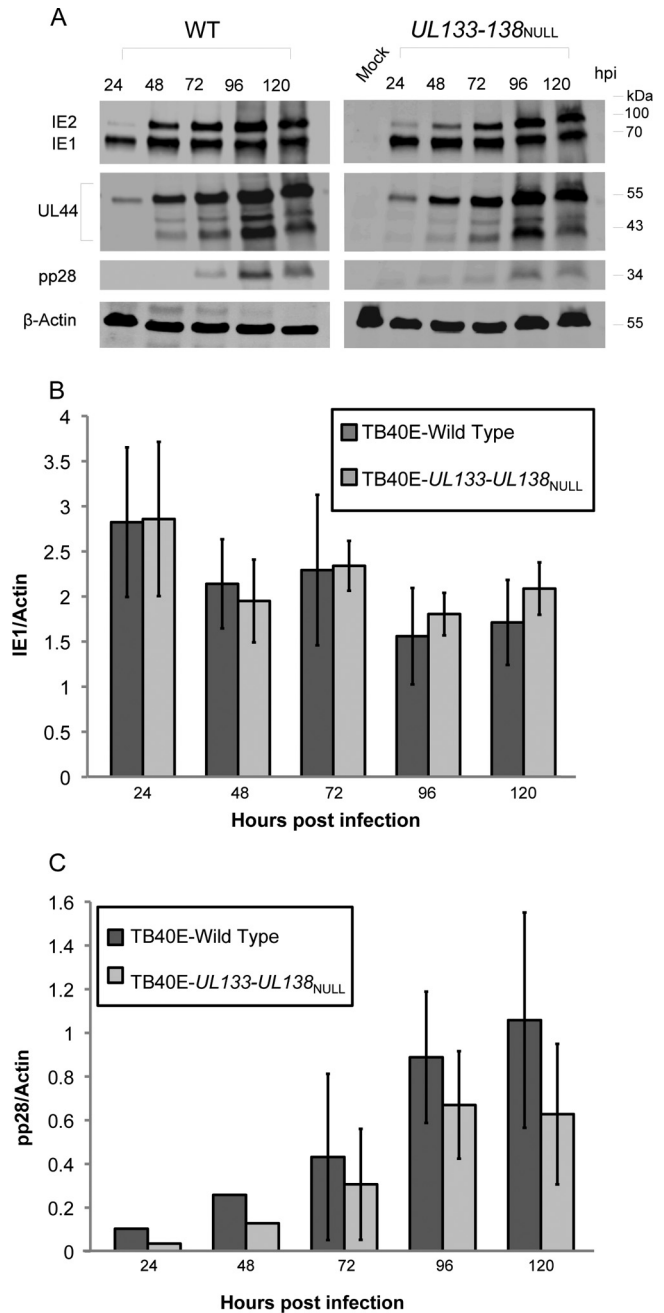


FIG 3 Accumulation of IE, early, and late proteins in infected HMVEC. HMVEC were infected with TB40E-WT or -*UL133-UL138*_{NULL} virus at an MOI of 2. (A) Protein lysates harvested over a time course were analyzed by immunoblotting using antibodies specific to the IE1 and IE2 proteins, the UL44 early protein, and the pp28 late protein. β -Actin serves as a loading control. Quantification of average IE1 (B) and pp28 (C) protein levels (normalized to actin) in at least four independent experiments is shown. The standard deviation is indicated. Student's *t* test indicates that the differences between WT and *UL133-UL138*_{NULL} virus at each of the time points for IE1 ($P \geq 0.2$) and pp28 ($P \geq 0.1$) are not significant.

cate that the defect in *UL133-UL138*_{NULL} virus replication is a late-phase defect.

***UL133-UL138* locus is required for production of mature virions in ECs.** To further characterize the late-phase defect associ-

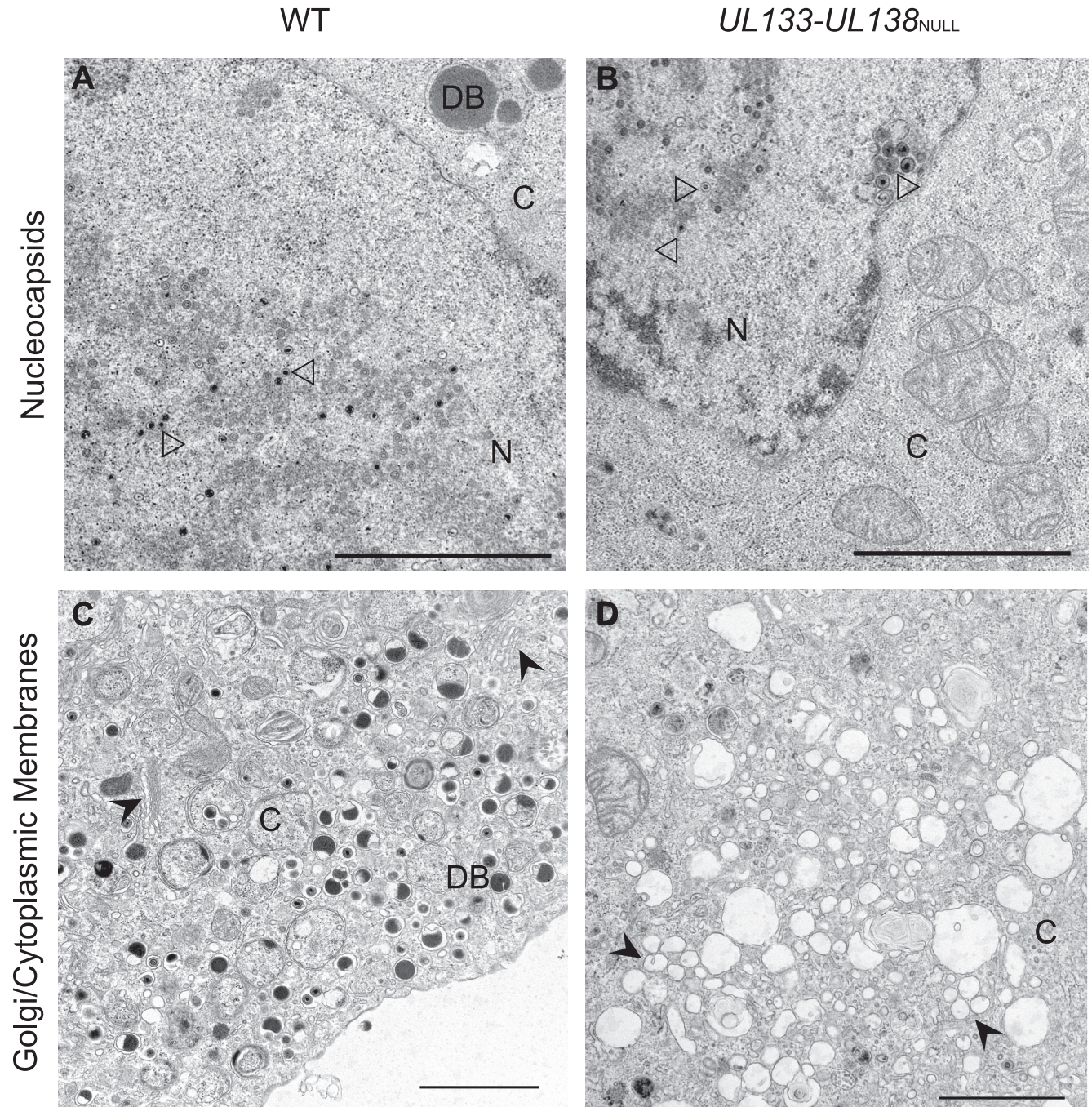


FIG 4 *UL133-UL138* is required for maintaining cytoplasmic membrane organization and for virion maturation. HMVEC were infected with WT or *UL133-UL138*_{NULL} virus at an MOI of 4. Cells were fixed, embedded, and sectioned for transmission electron microscopy at 5 dpi. Representative micrographs are shown to illustrate the accumulation of nucleocapsids (A and B), the organization of cytoplasmic membranes (C and D), the formation of MVBs (E and F), and the maturation of cytoplasmic virions (G and H). N and C indicate nucleus and cytoplasm, respectively; DB indicates dense bodies. Open arrowheads, nucleocapsids (A and B); filled arrowheads, Golgi stacks (C) or vesicles (D); filled arrows, MVBs (E and F); open arrows, cytoplasmic virus particles (G and H). Scale bars, 2 μ m (A to D) and 0.5 μ m (E to H).

ated with the production of infectious progeny in *UL133-UL138*_{NULL} virus infection (Fig. 1), we evaluated the formation of mature virions in infected ECs by transmission electron microscopy. HMVEC were infected with TB40E-WT or -*UL133-UL138*_{NULL} virus at an MOI of 4 and processed for electron microscopy at 5 dpi, a time when infectious virus is produced (Fig. 1

(60). The accumulation of viral nucleocapsids appeared normal in mutant-infected HMVEC relative to the WT infection (Fig. 4A and B); both empty and genome-containing nucleocapsids were apparent with similar frequencies in each infection. Furthermore, there were no significant differences apparent between WT and mutant infections in the organization of the nucleus or viral rep-

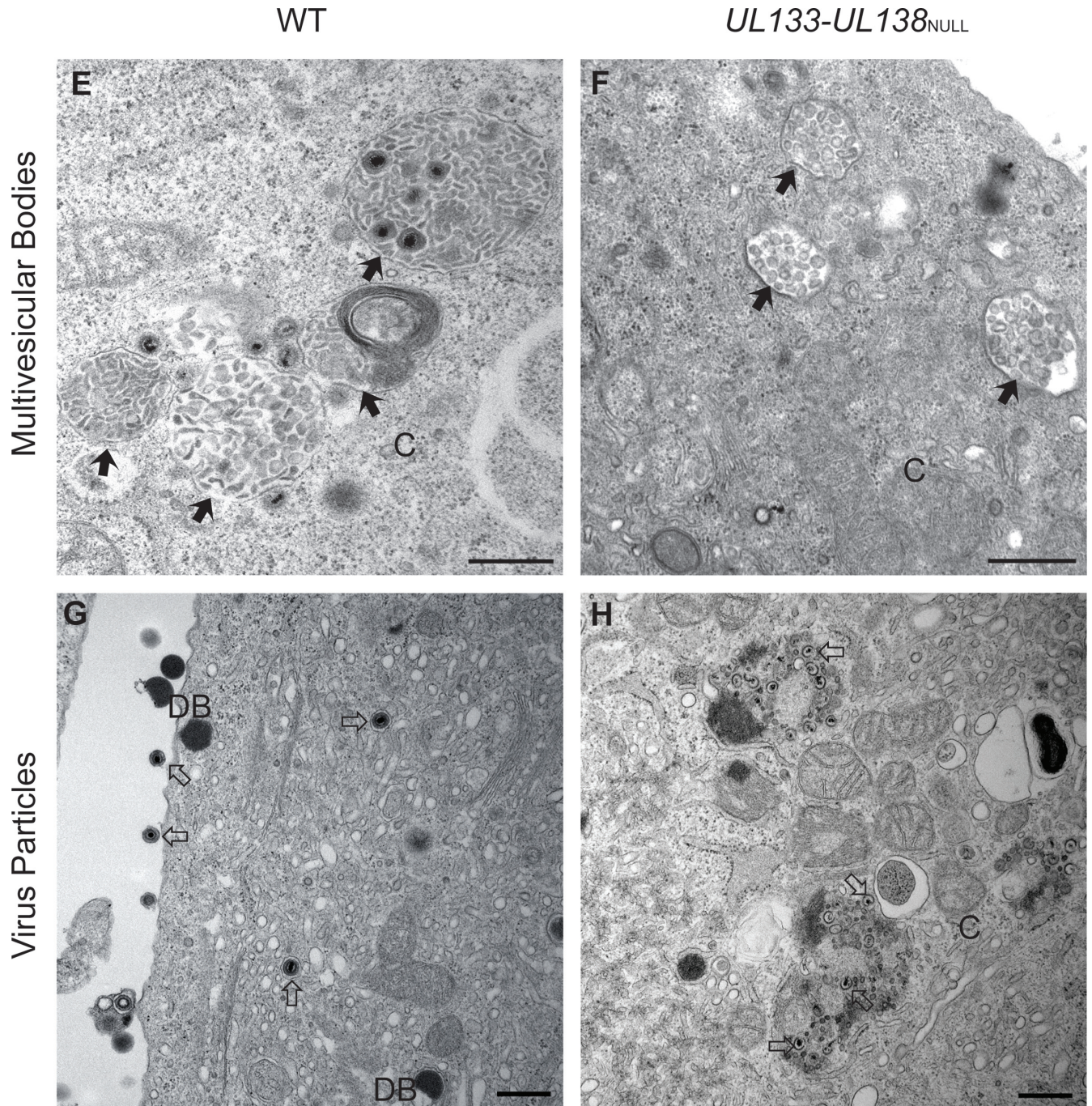


FIG 4 continued

lication centers. In stark contrast, significant differences between the WT and *UL133-UL138*^{NULL} viruses emerged in the cytoplasm. The most noteworthy differences exist in (i) the integrity of the Golgi complex and cytoplasmic membranes (Fig. 4C and D), (ii) the appearance of multivesicular bodies (MVBs) (Fig. 4E and F), and (iii) the presence of mature cytoplasmic virions (Fig. 4G and H).

Golgi stacks were readily apparent in the cytoplasm of every WT virus-infected cell examined (Fig. 4C). In addition, large numbers of membrane-bound, tegument-filled dense bodies (DBs) were observed in the cytoplasm of WT virus-infected cells.

In comparison, the cytoplasm of HMVEC infected with *UL133-UL138*^{NULL} virus contained large electron-lucent vesicles and no apparent Golgi stacks (Fig. 4D). Further, DBs were far less abundant. These data suggest that the *UL133-UL138* locus may be important for maintaining intracellular membrane organization and the formation of DBs during infection in ECs.

MVBs are late endosomes that play important roles in modulation of cell signaling, lipid and membrane protein turnover, antigen presentation, and the egress of some viruses (63). MVBs were induced by both WT and *UL133-UL138*^{NULL} virus infection

(Fig. 4E and F); MVBs were rarely observed in uninfected HMVEC (data not shown). The contents of the MVBs differed qualitatively between the WT and *UL133-UL138*_{NULL} virus infections. In the WT infection, MVBs often contained mature virions (tegumented and enveloped), whereas this was not the case in the mutant infection (Fig. 4E). In WT infection, mature virions were present inside MVBs, while immature virions were present at the periphery of MVBs, suggesting that MVBs may play a role in the final steps of virion maturation in these cells. Further, the vast majority of membrane vesicles inside MVBs present in the WT infection were flattened or compressed. These compressed vesicles were not observed in *UL133-UL138*_{NULL} virus-infected HMVEC (Fig. 4F), and the vesicles present retained a shape typically observed in MVBs.

In addition to the mature virions present in MVBs (Fig. 4E), WT infection in HMVEC produced individual enveloped, mature virions in the cytoplasm, and extracellular virions were present (Fig. 4G). In contrast, mature virions were far less abundant in the *UL133-UL138*_{NULL} virus infection (Fig. 4H), reflecting diminished viral yields (Fig. 1). Instead, untegumented and unenveloped particles were commonly observed and often clustered around spherical structures in the cytoplasm. Further, these virions had a “cracked” appearance. From our electron microscopy, we conclude that *UL133-UL138*_{NULL} virus infection is defective for processes in tegumentation of virus particles and secondary envelopment.

The *UL133-UL138* locus is not required for virus maturation in fibroblasts. Our result in ECs prompted analysis of TB40E *UL133-UL138*_{NULL} virus-infected fibroblasts to determine if there was any alteration in mature virion formation in these cells. In stark contrast to infection in ECs, fibroblasts infected with *UL133-UL138*_{NULL} virus produce WT yields of virus, suggesting that there is no defect for virus replication in the absence of the *UL133-UL138* locus (60). We analyzed fibroblasts infected with WT or *UL133-UL138*_{NULL} virus at an MOI of 2 at 5 dpi for the presence of mature virions by electron microscopy (Fig. 5). The cytoplasm of fibroblasts infected with *UL133-UL138*_{NULL} virus contained large numbers of mature virions with both tegument and envelope similar to those of WT infection in fibroblasts (Fig. 5A and B). In addition, Golgi stacks were readily apparent in both infections, and the overall architecture of the cytoplasm did not vary. These data highlight the unique EC-specific defect associated with *UL133-UL138*_{NULL} virus replication.

The assembly compartment is poorly formed in ECs infected with TB40E-*UL133-UL138*_{NULL} virus. In fibroblasts, HCMV infection results in a dramatic reorganization of cellular cytoplasmic membranes to form a juxtannuclear compartment commonly referred to as the assembly compartment (AC) (1, 64–70). Due to the altered morphology of the Golgi compartment and the lack of mature virions in ECs infected with *UL133-UL138*_{NULL} virus, we examined the formation of the AC. ECs were infected with TB40E-WT or -*UL133-UL138*_{NULL} virus at an MOI of 2. We examined the distribution of the late viral protein, pp28, and the *cis*-medial Golgi marker, GM130, in infected ECs by indirect immunofluorescence at 144 hpi (Fig. 6A and B). pp28 is known to localize to the AC and is an abundant tegument protein in the mature virion. GM130, marking Golgi membranes, is dramatically reorganized to the peripheral zone of the AC (66, 67, 69). In both WT and *UL133-UL138*_{NULL} virus infections, the characteristic Golgi stack-staining of GM130 was disrupted. In WT infection

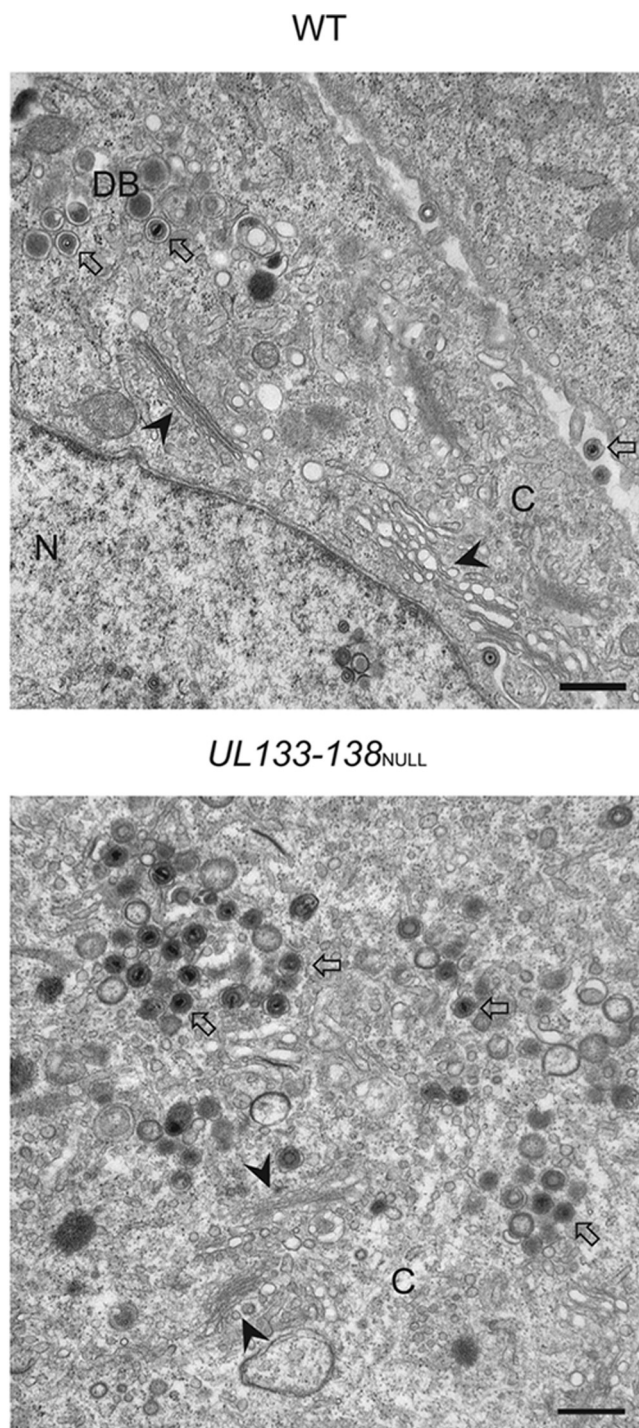


FIG 5 *UL133-UL138* is dispensable for mature virion formation in infected fibroblasts. MRC-5 fibroblasts were infected with WT (top panel) or *UL133-UL138*_{NULL} (bottom panel) virus at an MOI of 2. At 5 dpi, cells were fixed, embedded, and sectioned for transmission electron microscopy. Representative micrographs are shown to illustrate the presence of Golgi stacks and mature virions in each infection. N and C indicate nucleus and cytoplasm, respectively; DB indicates dense bodies. Filled arrowheads, Golgi stacks; open arrows, virions. Scale bar, 0.5 μ m.

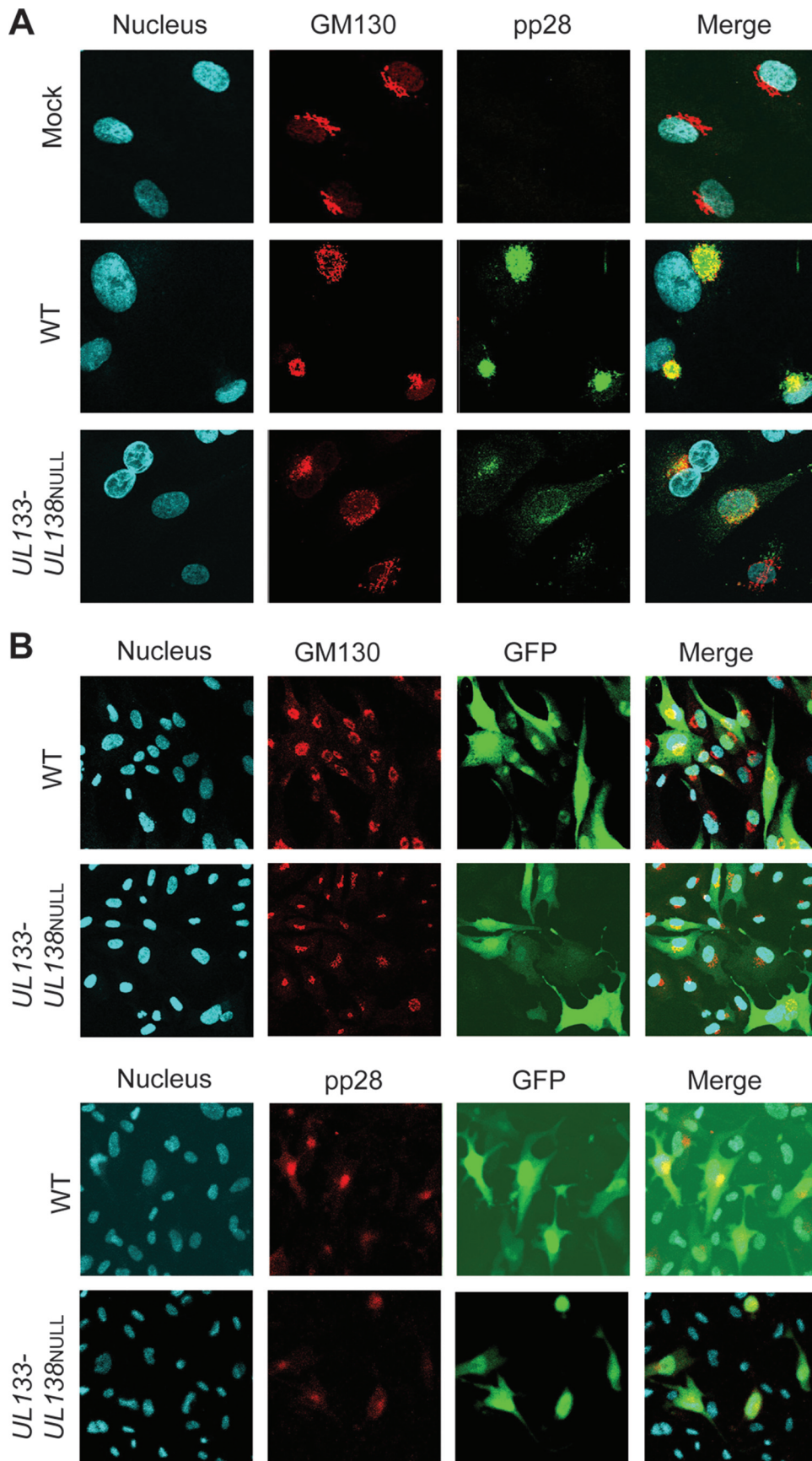


FIG 6 *UL133-UL138_{NULL}* virus-infected ECs fail to form assembly compartments. HMVEC were infected with WT or *UL133-UL138_{NULL}* virus at an MOI of 2 or mock infected. At 144 h postinfection, cells were processed for indirect immunofluorescence using antibodies specific to the GM130 Golgi marker or pp28 viral protein. The nuclei are indicated by DAPI staining. Localization was visualized by confocal microscopy using a Zeiss 510 Meta Confocal microscope. Panel B contains low-magnification fields of cells to demonstrate the range of AC morphology in each infection. Magnification, $\times 60$ (A) and $\times 40$ (B).

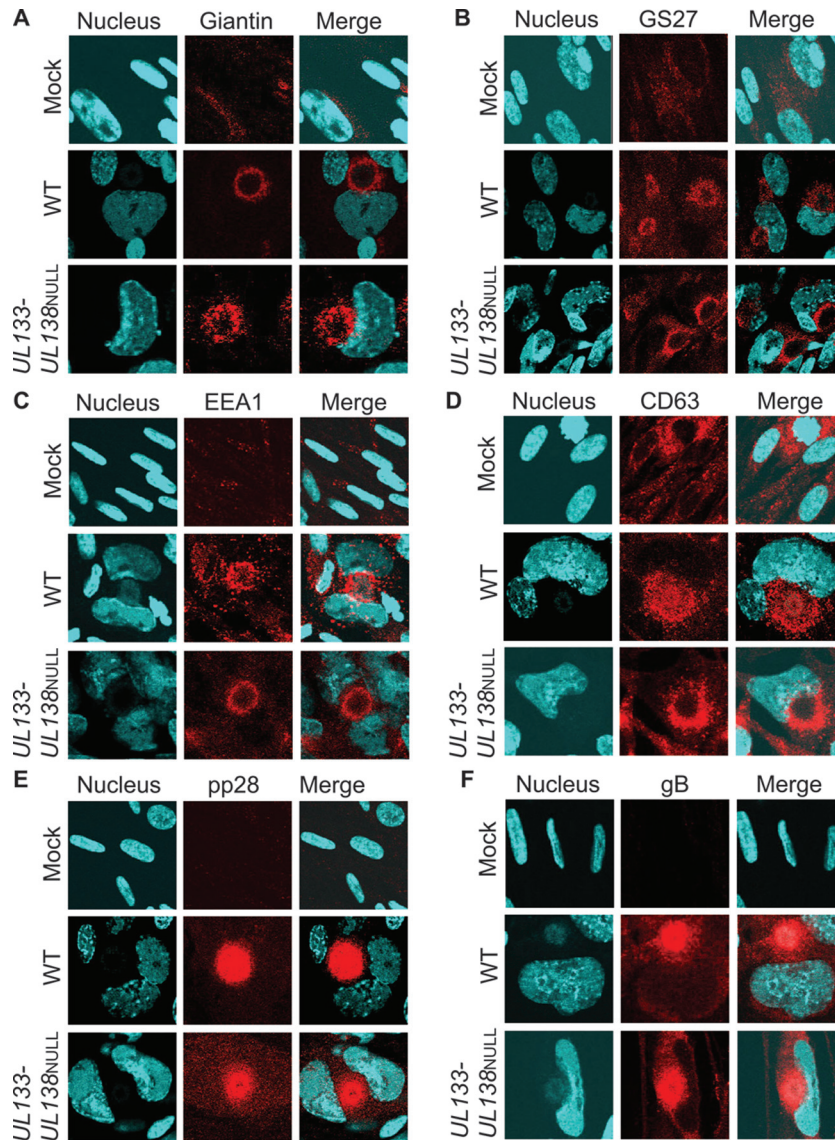


FIG 7 Assembly compartment formation in fibroblasts is similar for WT and *UL133-UL138*_{NULL} virus infection. MRC-5 fibroblasts were infected with WT or *UL133-UL138*_{NULL} virus at an MOI of 2 or mock infected. At 96 hpi, cells were processed for indirect immunofluorescence using antibodies specific to giantin (A), GS27 (B), EEA1 (C), CD63 (D), pp28 (E), or gB (F). Infection of the cells imaged was determined by GFP fluorescence (data not shown). The nuclei are indicated by DAPI staining. Localization was visualized by confocal microscopy using a Zeiss 510 Meta Confocal microscope. Magnification, $\times 60$.

of HMVEC, the characteristic ring-like structure stained by GM130 indicated the formation of ACs, similar to our results described in fibroblasts (66, 67). In the WT infection, pp28 exhibited remarkable overlap in its staining with GM130, characteristic of the formation of late-phase ACs. In contrast, GM130 and pp28 staining was dispersed throughout the cytoplasm of ECs infected with *UL133-UL138*_{NULL} virus. While there was some overlap in GM130 and pp28 staining in *UL133-UL138*_{NULL} virus-infected HMVEC, colocalization was reduced compared to that of the WT infection. Low-magnification images illustrate the range of differences in staining observed in a field of ECs infected with either the WT or *UL133-UL138*_{NULL} virus (Fig. 6B). In the analysis of pp28 staining in more than 100 cells, a focal AC was observed in 85% of WT-infected ECs but in only 34% of *UL133-UL138*_{NULL} virus-infected ACs. These results indicate that ACs form inefficiently in ECs infected with *UL133-UL138*_{NULL} virus.

To further investigate the role of the *UL133-UL138* locus in the formation of ACs, we analyzed cellular Golgi markers (giantin and GS27), the early endosomal marker (EEA1), the MVB marker (CD63), viral pp28, and viral glycoprotein B (gB) in both MRC-5 fibroblasts (Fig. 7) and HMVEC (Fig. 8). Each of these protein markers localizes to the AC in WT infection in fibroblasts (66). MRC-5 cells or HMVEC infected with TB40E-WT or *-UL133-UL138*_{NULL} virus at an MOI of 2 were processed for indirect immunofluorescence at 96 hpi in fibroblasts (Fig. 7) and at 144 hpi in HMVEC (Fig. 8). ECs were processed at a later time postinfection due to slower kinetics of infection in these cells. Infected cells were identified by GFP fluorescence (data not shown). gB and pp28 staining was evaluated as a marker of ACs.

In both WT and *UL133-UL138*_{NULL} virus-infected fibroblasts, giantin (Fig. 7A), GS27 (Fig. 7B), EEA1 (Fig. 7C), and CD63 (Fig. 7D) were reorganized by infection to virus-induced ACs as previ-

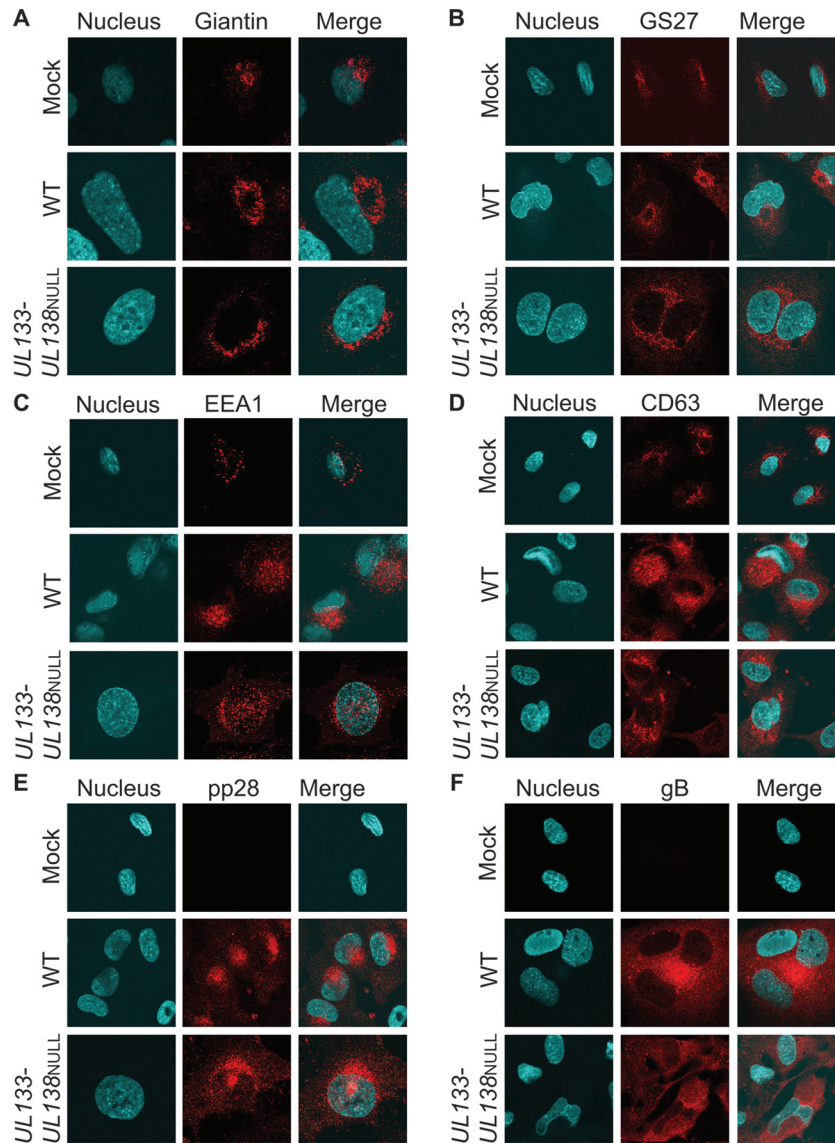


FIG 8 Analysis of cellular membrane organization and viral assembly compartment markers in WT and *UL133-UL138*_{NULL} virus infection in ECs. HMVEC were infected with WT or *UL133-UL138*_{NULL} virus at an MOI of 2 or mock infected. At 144 hpi, cells were processed for indirect immunofluorescence using antibodies specific to giantin (A), GS27 (B), EEA1 (C), CD63 (D), pp28 (E), or gB (F). Infection of the cells imaged was determined by GFP fluorescence (data not shown). The nuclei are indicated by DAPI staining. Localization was visualized by confocal microscopy using a Zeiss 510 Meta Confocal microscope. Magnification, $\times 60$.

ously described (66, 67). The characteristic ring-like structure of the AC was apparent for each of the markers staining cellular cytoplasmic structures. The localization of pp28 and gB to assembly compartments in *UL133-UL138*_{NULL} virus-infected fibroblasts was similar to that of the WT infection although in some cases the staining for these proteins was slightly more diffuse relative to that in the WT infection. These results suggest that the formation of ACs in fibroblasts does not require the *UL133-UL138* locus.

Similar to WT infection in fibroblasts, cytoplasmic membrane markers were reorganized to form ACs in WT-infected ECs (Fig. 8A to D). Further, pp28 and gB staining was indicative of ACs in the WT infection (Fig. 8E and F). Consistent with the data shown in Fig. 6, HMVEC infected with *UL133-UL138*_{NULL} virus displayed poorly formed ACs, and all cellular membrane markers were more diffusely localized (Fig. 8A to D) relative to WT infec-

tion in fibroblasts. This diffuse distribution of membrane markers is consistent with the loss of Golgi stacks and increased numbers of large cytoplasmic vesicles observed by electron microscopy (Fig. 4). Therefore, our data indicate a role for the *UL133-UL138* proteins in efficient formation of the AC for virus assembly.

DISCUSSION

The endothelium represents an important target for HCMV infection as it contributes to viral dissemination and pathogenesis. HCMV determinants important for postentry endothelial tropism have remained elusive and represent an important point of control. Our work reveals a novel EC-specific function of the *UL133-UL138* locus encoded within the *ULb'* region of the HCMV genome in maintaining intracellular membrane organization and in the formation of the AC for mature virus production.

These results identify the first virus-coded determinants important for late stages of viral infection in ECs. The EC-specific function of the *UL133-UL138* locus was revealed by a specific defect in *UL133-UL138*_{NULL} virus replication in a variety of EC types but not in fibroblasts or epithelial cells (Fig. 1) (60). There was no defect in entry or in immediate-early and early gene expression and less than a 2-fold defect in late gene expression in ECs infected with *UL133-UL138*_{NULL} virus relative to the wild-type infection (Fig. 2 and 3). Strikingly, virions formed during *UL133-UL138*_{NULL} virus infection in ECs lacked tegument and secondary envelopes (Fig. 4). The defect in virion maturation observed in *UL133-UL138*_{NULL} virus-infected cells may be secondary to a defect in intracellular membrane organization and the failure to form an AC for virus maturation (Fig. 6 and 8).

Herpesviruses acquire their tegument and a host-derived envelope during a complex process of egress involving a variety of membrane-bound cellular compartments (71–74). HCMV is unique among the herpesviruses in that it induces the formation of a juxtannuclear virus factory, or AC, comprised of secretory/trafficking membranes, including those of the Golgi complex, early endosomes, lysosomes, and MVBs (1, 64–70). Many late viral proteins that are components of the mature virion, including the viral glycoproteins (i.e., gB), pp28, and pp150, localize to the AC (75). As such, the AC is thought to facilitate the acquisition of tegument and secondary envelopes in the maturation of HCMV particles during the late, postnuclear stages of infection. While the AC has been described predominantly in fibroblasts, the conventional cell culture model for HCMV research, ACs are formed in HCMV-infected ECs (Fig. 6 and 8). Cellular secretory markers such as GM130, giantin, GS27K, EEA1, and CD63 localize to the AC in WT HCMV-infected ECs (Fig. 6 and 8) in a pattern and organization similar to those described in fibroblasts (66, 67, 69). Strikingly, the *UL133-UL138* locus was required for the formation of the AC in ECs but not in fibroblasts. The cell-type-specific requirement for the *UL133-UL138* locus in the formation of the AC likely reflects specific interactions between *UL133-UL138* proteins and cellular factors that may function in membrane transport including, but not limited to, the endosomal sorting complex required for transport (ESCRT) machinery.

Host vesicles and membrane-bound organelles reorganized to become the AC are important for HCMV envelopment (71–73). MVBs (CD63-positive) and their possible role in constitutive or alternative pathways of virus maturation are of particular interest (76). A role for MVBs in HCMV egress is supported by the observations that MVBs were induced by infection, that they reorganized into the AC late in infection, as indicated by CD63 staining (Fig. 8) (67), and that they often contained mature virions in WT-infected ECs (Fig. 4E). Consistent with a possible role of the *UL133-UL138* locus in mediating intracellular membrane organization, striking differences were observed in MVBs induced by the WT and *UL133-UL138*_{NULL} virus infections in ECs. Vesicles contained within MVB were often flattened or compressed in ECs infected with WT but not *UL133-UL138*_{NULL} virus (Fig. 4E and F). The observation of flattened vesicles within MVBs in WT virus-infected ECs taken together with the lack of Golgi stacks in *UL133-UL138*_{NULL} virus-infected cells suggests a possible role for *UL133-UL138* proteins in “stacking” membranes.

In fibroblasts, MVBs and ESCRT machinery play an important role in HCMV egress (76, 77). MVBs and ESCRT machinery are associated with a number of cellular processes that may impact

outcomes of infection, including signaling, membrane and lipid turnover, and cellular proliferation and polarization (78). In addition to HCMV, MVBs and ESCRT machinery have been shown to play a role in the assembly and egress of numerous viruses, including Marburg virus (79), retroviruses (80–83), hepatitis B virus (84), herpes simplex virus 1 (HSV-1) (85–88), Epstein-Barr virus (89), and human herpesvirus 6 (77, 90). Blocking MVB formation by targeting either Vps4 or Vps24 of the ESCRT-III complex during HSV infection results in a dramatic reduction in the yield of cell-associated and cell-free virus, depending on the cell type infected (85, 86, 88). In HIV infection, the virus buds directly into MVBs in monocytic cells, whereas MVBs are recruited to the plasma membrane to become a site of budding in lymphocytic cells (80). These studies, among others, underscore the central importance of MVBs to virus envelopment and egress. Indeed, MVBs may play different roles in egress in a cell-type- and infection-dependent manner. Further work is required to determine the role of MVBs and the ESCRT machinery in HCMV egress from ECs and how the *UL133-UL138* proteins may contribute to this process through EC-specific interactions.

While it is not fully understood how the HCMV virion acquires its tegument during egress, the tegument is critical for at least three important functions. These include the packaging and structural stability of nucleocapsids, mediating interactions necessary for final envelopment and egress, as well as playing a vital role in initiating the second round of infection (75). While the *UL133-UL138* proteins localize to Golgi membranes (60, 61), these proteins are not thought to be packaged in progeny virions (61). However, the *UL133-UL138* locus appears to contribute to the acquisition of the virus tegument. Further work is required to understand if the failure of virions to acquire tegument is a direct function of these proteins or a consequence of the failure to reorganize intracellular membranes into an AC. Viruses lacking the HCMV gene, *UL71*, exhibit a similar phenotype to what we have observed during infection with *UL133-UL138*_{NULL} virus (76). Infection with a *UL71* mutant virus alters MVB morphology, mislocalizes pp28, pp150, and gB, alters the morphology of the AC, and impairs secondary envelopment. Interestingly, the *UL71* mutant virus phenotype is apparent only in fibroblasts and not ECs. Taken together with our study, these results suggest that HCMV has evolved similar but distinct mechanisms for viral egress in different cell types.

Our study defines a novel EC-specific role for the *UL133-UL138* locus in mediating intracellular membrane reorganization for egressing virus to acquire both tegument and secondary envelope. Ongoing work is focused on determining how the phenotypes observed in ECs segregate between the four genes encoded within the locus. Preliminary studies indicate that at least two of the *UL133-UL138* proteins are required for the EC-specific functions we have ascribed to the *UL133-UL138* locus in this study (F. Bugchio and F. Goodrum, unpublished results). We previously demonstrated a role for the *UL133-UL138* locus, specifically pUL133 and pUL138, in promoting a latent infection in CD34⁺ hematopoietic progenitor cells (HPCs). It is not yet clear how these new functions of the *UL133-UL138* locus in ECs may contribute to infection in HPCs. Defining the mechanisms by which the individual proteins encoded by the *UL133-UL138* locus contribute to the replication in ECs and latency in CD34⁺ HPCs is a primary focus of our ongoing research. The relationship of these four proteins to one another and their role in viral replication and

persistence will undoubtedly prove complex. The existence of viral genes important for viral replication in ECs opens up new avenues for targeting virus replication to ameliorate vascular-related pathologies associated with HCMV infection, including atherosclerosis, restenosis, and chronic graft rejection.

ACKNOWLEDGMENTS

We thank William Day of the Arizona Research Laboratories and Patricia Jansma of the Molecular and Cellular Biology Imaging Facility for their expertise and assistance with transmission electron and confocal microscopy, respectively. We acknowledge Paula Campbell and the AZCC/ARL Division of Biotechnology Cytometry Core Facility for expertise in flow cytometry. We gratefully acknowledge the gift of antibodies from Thomas Shenk, William Britt, Jay Nelson, Michael Nevels, and Samuel Campos. We thank Jean Wilson for helpful discussions. We acknowledge M. Umashankar, K. Caviness, and P. Zagallo for critical reading of the manuscript.

This work was supported by Public Health Service grant AI079059 from the National Institute of Allergy and Infectious Diseases and by a Cancer Center Support grant (CA020374). F.G. is a Pew Scholar in the Biomedical Sciences, supported by the Pew Charitable Trusts.

The content of this paper is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute, the National Institute of Allergy and Infectious Diseases, the National Institutes of Health, or The Pew Charitable Trusts.

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