

Cellular defense against latent colonization foiled by human cytomegalovirus UL138 protein

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Intrinsic immune defenses mediated by restriction factors inhibit productive viral infections. Select viruses rapidly establish latent infections and, with gene expression profiles that imply cell-autonomous intrinsic defenses, may be the most effective immune control measure against latent reservoirs. We illustrate that lysine-specific demethylases (KDMs) are restriction factors that prevent human cytomegalovirus from establishing latency by removing repressive epigenetic modifications from histones associated with the viral major immediate early promoter (MIEP), stimulating the expression of a viral lytic phase target of cell-mediated adaptive immunity. The viral UL138 protein negates this defense by preventing KDM association with the MIEP. The presence of an intrinsic defense against latency and the emergence of a cognate neutralizing viral factor indicate that “arms races” between hosts and viruses over lifelong colonization exist at the cellular level.

INTRODUCTION

Intrinsic defenses (1, 2) are cell-autonomous components of the immune system that protect individual cells from viral attack. Mediated by constitutively expressed proteins called restriction factors, they can rapidly act to inhibit viral infections at very early stages. Examples of intrinsic defenses include APOBEC3G (3), TRIM5 α (4), and SAMHD1 (5, 6), which inhibit reverse transcription of HIV, and the promyelocytic leukemia (PML) (7), Sp100 (8), and Daxx (9) proteins that inhibit herpesvirus immediate early (IE) transcription. A hallmark of known intrinsic defenses is the presence of a viral countermeasure that inactivates them. These include the viral capsid, Vif, and Vpx proteins (10) for HIV and the ICP0 (11), IE1 (2), and pp71 proteins (12) for herpesviruses.

All known intrinsic defenses inhibit the productive, lytic replication cycles that generate infectious progeny virions that allow for viral spread within and between hosts. They do so by inhibiting an essential viral process. However, HIV and herpesviruses also establish latent infections (13, 14), during which infectious virions are not generated. Latent virus can be reactivated to a productive stage that releases transmissible progeny. Latently infected cells present a barrier to viral clearance because they do not express pathogen-associated molecular patterns that prompt innate immune responses and are not eliminated by either the humoral or cell-mediated arms of the adaptive immune response, rendering them invisible to much of the immune system. The depth (number of latently infected cells) and complexity (number of different genotypes) of latent reservoirs are likely important for successful reactivations in immune-primed hosts. Prevailing dogma assumed that a lengthy viremic period after primary infection of a host organism was required to generate a latent reservoir. However, recent experimental and clinical data indicate that, at least for HIV, functional latent reservoirs are generated within days or even hours after primary infection (15, 16). This represents a time frame out of reach of adaptive or innate immune responses but susceptible to intrinsic immunity.

HIV identically initiates productive and latent infections through reverse transcription of their RNA genomes into DNA proviruses

followed by integration; hence, intrinsic defenses against lytic HIV also inhibit the establishment of latency. In contrast, herpesviruses such as herpes simplex virus type 1 (HSV-1) and human cytomegalovirus (HCMV) have DNA genomes, do not integrate, and initiate productive infections or establish latency through unique and opposing mechanisms. Productive infections initiate when viral IE transcription is activated, whereas latent infections are established when IE transcription is silenced (17). For HCMV, productive infections initiate in terminally differentiated cells such as fibroblasts when the virion-delivered pp71 tegument protein migrates to the nucleus and induces the degradation of the cellular Daxx protein (9). In the absence of pp71, Daxx mediates an intrinsic defense against HCMV that silences viral IE transcription by instituting a repressive chromatin structure (18) on the viral major immediate early promoter (MIEP). The Daxx intrinsic defense can be artificially inactivated by histone deacetylase (HDAC) inhibitors such as valproic acid (VPA) or by Daxx knockdown with RNA interference (19, 20).

When HCMV establishes latency within incompletely differentiated cells of the myeloid lineage, tegument-delivered pp71 remains in the cytoplasm, allowing the Daxx/HDAC-mediated intrinsic defense to silence viral IE transcription. Transient or constitutive Daxx knockdown or treatment with VPA permits IE1 transcript and protein accumulation in NT2 cells, THP-1 cells, primary CD34⁺ cells, and embryonic stem cells (ESCs) infected with the HCMV strain AD169 during the first day of infection (19–21). Without such treatments, IE1 transcript or protein accumulation does not occur in this time frame (19–21). This is a unique example where a virus does not neutralize an intrinsic defense against productive infection but actually uses it in certain cell types to promote cellular latency and viral persistence. Inhibiting IE transcription during the establishment of latency is imperative because the major IE protein, IE1, promotes robust productive replication (22), which is antithetical to latency, and is a prominent HCMV antigen recognized by CD8⁺ cytotoxic T cells (23). Consistent with the inhibition of IE gene expression, latent viral chromatin shows epigenetic marks of transcriptional repression (24–26) including hypoacetylated histones, trimethylation of histone H3 lysine 27 (H3K27me3), and association with heterochromatin protein 1 (HP1), which is mediated by di- or trimethylation of histone H3 lysine 9 (H3K9me2/3).

For high-passage laboratory strains of HCMV such as AD169, the Daxx/HDAC defense is the only known transcriptional silencer of IE1

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during experimental latency in primary CD34⁺ hematopoietic progenitor cells. However, we showed that clinical HCMV strains (such as TB40/E and FIX) that more closely mimic naturally circulating viruses and encode more genes than AD169 (27) impart at least one additional restriction against IE1 transcription during latency (20), such that it cannot be rescued by VPA. This demonstration that inactivating the Daxx-mediated defense is insufficient to prevent clinical strains from establishing latency was confirmed by two independent groups (28, 29). Differences in myeloid tropism between laboratory-adapted and clinical strains cannot account for the observed differences in virus transcriptional regulation because FIX delivers tegument to only 1.6-fold more primary CD34⁺ cells than does AD169 (20), and TB40/E delivers thrice as many genomes to primary monocyte cultures than does AD169 (30). For comparison, the difference in endothelial cell tropism between clinical strains and AD169 is 100-fold (31). Our desire to identify this additional restriction found only in clinical strains led us to UL138 (32), a clinical strain-specific gene that is implicated in the establishment or maintenance of latency because its deletion causes mutant viruses to more frequently generate infectious progeny during the latent period (33). Because viral IE gene products promote productive replication, we reasoned that infectious progeny may be produced by UL138-deficient viruses because they fail to fully silence viral IE transcription during latency. We show here that UL138 is indeed capable of repressing IE1 transcription during experimental latent HCMV infections in CD34⁺ cells.

We also determined the unique and intriguing mechanism through which UL138 silences HCMV IE1 transcription during latency. Knowing that UL138 maintained function in the presence of VPA and was thus unlikely to affect histone acetylation, we focused on histone methylation. We discovered that UL138 prevented the cellular lysine demethylases (KDMs) KDM1A (LSD1) and KDM6B (JMJD3) and the KDM-interacting protein CtBP1 from associating with the MIEP and inhibited the removal of repressive histone methylations at this locus. Provocatively, UL138 does not act by directly suppressing HCMV gene expression. Rather, it inhibits cellular proteins from activating viral IE1 transcription in undifferentiated myeloid cells that would prevent latency establishment. Thus, we describe the cellular system that promotes this activation as an intrinsic defense specific for HCMV latency that is neutralized by UL138. Cell-autonomous protection against HCMV latency illustrates that our immune system is geared to deal with viruses that rapidly generate latent reservoirs. HCMV inactivates this defense, reinforcing that measures to prevent latency establishment are viable and desired antiviral strategies.

RESULTS

HCMV UL138 represses viral IE1 transcription

Although UL138 failed to inhibit an MIEP reporter in terminally differentiated cells (34), we found that it was able to repress an MIEP reporter (fig. S1A) but not a reporter driven by the early promoter from simian virus 40 (fig. S1B) in THP-1 cells, a model for latent infections used by us (19, 20, 35, 36) and used widely by other groups (29, 32, 37–50). IE1 transcription is initially silenced in THP-1 cells, as it is in primary CD34⁺ cells (20). Immediately upon infection by AD169, IE transcription is promoted by either VPA treatment or Daxx knock-down in both THP-1 and primary CD34⁺ cells (19, 20, 35, 36, 51). In contrast, IE1 transcription immediately upon infection by the clinical

strain FIX or TB40/E is not promoted by VPA treatment in either THP-1 or primary CD34⁺ cells (20). The repressive methylation mark H3K27me3 is found at the MIEP in AD169-infected THP-1 cells (46) (see below) and primary CD34⁺ cells (see below). Because the published data exploring IE1 transcriptional patterns and MIEP epigenetics in THP-1 and primary CD34⁺ cells are indistinguishable, we and others use both as model cell systems for studying HCMV latency. IE1 activated the MIEP in THP-1 cells (fig. S1A), implying that it remains capable of promoting productive infection in incompletely differentiated myeloid cells as it does in differentiated cells.

To determine whether UL138 also repressed the MIEP in the context of viral infection, we generated an AD169 derivative (AD-138HA) that encodes an epitope-tagged UL138 near its vestigial locus that is expressed from its native promoter (Fig. 1A and fig. S2A). Expressing a clinical strain allele in AD169 has been used previously to restore a clinical strain-specific phenotype (52), proving that the AD169 strain is a powerful tool for such add back experiments. In fibroblasts, AD-138HA grew to similar titers (fig. S2B) and expressed representative IE (IE1), early (UL44), and late (pp28) genes with similar kinetics and to similar levels as wild-type AD169 (fig. S2C). AD-138HA expressed UL138 mRNA (fig. S2D) that generated a full-length epitope-tagged UL138 protein (fig. S2C) that colocalized with the Golgi marker GM130 in fibroblasts (fig. S3A), THP-1 cells (fig. S3B), and primary CD34⁺ cells (fig. S3C) as expected (34). AD-138HA also expressed UL138 mRNA in CD34⁺ cells (fig. S4A) into which it entered as efficiently as AD169 based on quantitative examination of viral genome delivery (fig. S4B). We conclude that a native latent promoter inserted near its natural genomic position in a recombinant AD169 genome is capable of expressing a transgene during both lytic infection and latency.

Upon infection of CD34⁺ cells by either AD169 or AD-138HA, the lytic transcripts for IE1 (Fig. 1, B and C) were found at levels between 2000- and 4500-fold lower than those found in lytically infected fibroblasts. Lytic transcripts for the early/late gene pp65 in CD34⁺ cells were found at levels more than 100-fold lower than those in fibroblasts (Fig. 1B and fig. S5A), but the latent transcripts LUNA and B2.7 RNA were expressed (Fig. 1B), indicating that both viruses established latency. For AD169, the lytic IE1 gene remains silent and the latent LUNA transcript continues to be detected for at least 72 hours in CD34⁺ cells (fig. S5B), evidence that these cells remain in a latent state. As reported previously by Saffert *et al.* (20), VPA prevented the silencing of AD169 IE gene expression by the Daxx/HDAC intrinsic immune defense and allowed for the accumulation of IE1 transcripts in CD34⁺ cells (Fig. 1, D and E). However, VPA failed to permit accumulation of IE1 transcripts in AD-138HA-infected CD34⁺ cells (Fig. 1, D and E), indicating that this recombinant virus had established latency even when the Daxx/HDAC defense was inactivated, mimicking the phenotype of clinical strain viruses (20).

Similar results were obtained in both THP-1 cells and ESCs. In THP-1 cells, the lytic transcripts for IE1 (Fig. 1, B and C) were found at levels more than 500-fold lower than those found in lytically infected fibroblasts. Lytic transcripts for the early/late gene pp65 in THP-1 cells were found at levels more than 100-fold lower than those in fibroblasts (Fig. 1B and fig. S5A), but the latent transcripts LUNA and B2.7 RNA were expressed (Fig. 1B), indicating that both viruses established latency. Latent transcripts are more abundant in THP-1 cells than in CD34⁺ cells, likely due to increased infectivity. LUNA transcripts appear less abundant in the presence of UL138 in both THP-1 and CD34⁺ cells. LUNA is known to increase the expression of UL138

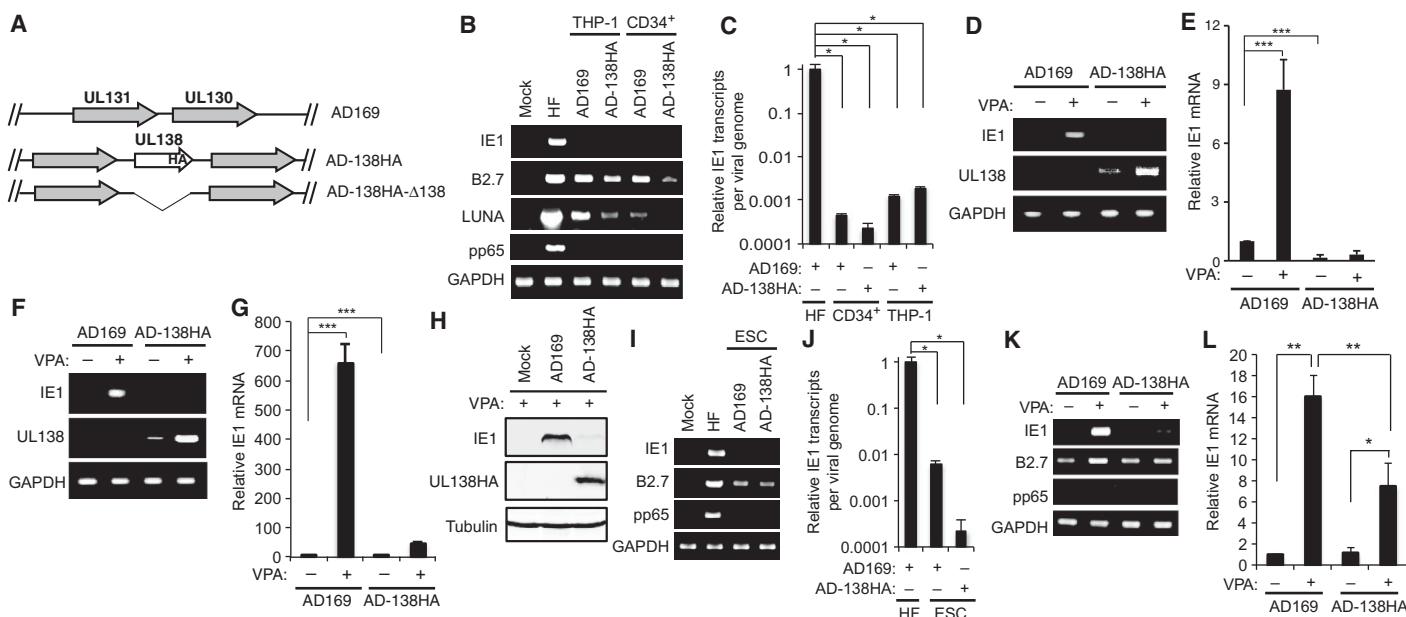


Fig. 1. UL138 inhibits VPA-responsive IE1 expression during HCMV latency. (A) Schematic of recombinant AD169 viruses encoding UL138 (see also fig. S2A). (B) RNA from THP-1 and primary CD34⁺ cells infected at a multiplicity of infection (MOI) of 1 with the indicated viruses for 18 or 24 hours, respectively, was analyzed for transcript expression of the indicated gene by reverse transcription polymerase chain reaction (RT-PCR). RNA from normal human dermal fibroblasts (NHDFs) infected with AD-138HA at an MOI of 1 for 24 hours serves as a positive control. (C) RNA and DNA from samples in (B) were analyzed by quantitative RT-PCR (qRT-PCR) or qPCR, respectively, and normalized to cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Viral transcripts were normalized to viral DNA and are shown relative to that of NHDFs (HF). (D and E) CD34⁺ cells infected at an MOI of 1 for 24 hours in the absence (–) or presence (+) of VPA (1 mM) analyzed for transcript expression by RT-PCR (D) or qRT-PCR (E). (F to H) THP-1 cells infected at an MOI of 3 for 18 hours in the absence (–) or presence (+) of VPA analyzed for transcript expression by RT-PCR (F) or qRT-PCR (G) and protein expression by Western blot (H). (I) ESCs infected with the indicated virus at an MOI of 3 for 24 hours analyzed for transcript expression of the indicated gene by RT-PCR. (J) ESCs infected as in (I) were analyzed for IE1 transcripts per viral genome as in (C). (K) ESCs infected with the indicated virus at an MOI of 3 for 24 hours in the absence (–) or presence (+) of VPA analyzed for transcript expression of the indicated gene by RT-PCR. (L) Samples from (K) were analyzed by qRT-PCR. Data are means ± SEM from at least three independent experiments. **P* < 0.05, ***P* < 0.01, or ****P* < 0.001 by Student's *t* test.

(53). It is possible that a feedback relationship exists (that is, UL138 inhibits LUNA expression). For AD169, the lytic IE1 gene remains silent and the latent LUNA transcript continues to be detected for at least 72 hours in THP-1 cells (fig. S5C), evidence that these cells remain in a latent state. As reported previously by Saffert and Kalejta (19), VPA prevented the silencing of AD169 IE gene expression by the Daxx/HDAC intrinsic immune defense and allowed for the accumulation of IE1 transcripts (Fig. 1, F and G) and protein (Fig. 1H) in THP-1 cells. However, VPA failed to permit accumulation of IE1 transcripts or protein in AD-138HA-infected THP-1 cells (Fig. 1, F to H).

In ESCs, the lytic transcripts for IE1 (Fig. 1, I and J) were found at levels ranging from 150- to 5000-fold lower than those found in lytically infected fibroblasts. Lytic transcripts for the early/late gene pp65 in ESCs were found at levels between 60- and 1000-fold lower than those in fibroblasts (Fig. 1I and fig. S5D), but the latent transcript B2.7 RNA was expressed (Fig. 1I), indicating that both viruses established latency. For AD169, the lytic IE1 gene remains silent and the latent LUNA and B2.7 RNA transcripts continue to be detected for at least 72 hours in ESCs (fig. S5E), evidence that these cells remain in a latent state. By 168 hours, IE1 transcripts were detected in ESCs (fig. S5E) as expected because this virus strain has difficulty maintaining latency (21, 33). LUNA transcripts also increase at this time, a finding not unexpected because LUNA is also expressed during lytic infection (54). Thus, we suspect that by 168 hours, AD169 is productively replicating

in ESCs. As reported previously by Penkert and Kalejta (21), VPA prevented the silencing of AD169 IE gene expression by the Daxx/HDAC intrinsic immune defense and allowed for the accumulation of IE1 transcripts (Fig. 1, K and L) in ESCs. However, VPA failed to permit accumulation of IE1 transcripts in AD-138HA-infected ESCs (Fig. 1, K and L). Although all cell types showed similar results, IE1 transcriptional repression was strongest in primary CD34⁺ cells, an unsurprising result because these represent the natural site of latency in HCMV-positive people.

An AD-138HA derivative in which UL138 was deleted (AD-138HA-Δ138; Fig. 1A and fig. S2A) produced identical gene expression signatures to AD-138HA in fibroblasts (fig. S6, A and B). AD-138HA-Δ138 produced identical gene expression signatures to wild-type AD169 in CD34⁺ cells (fig. S6, C and D) and THP-1 cells (fig. S6E). Therefore, UL138 is required for the phenotype of AD-138HA. Combined with the reporter data, we conclude that UL138 is sufficient to silence viral IE1 transcription in an HDAC-independent manner during the establishment of HCMV latency.

Clinical strains encode factors in addition to UL138 that contribute to latency establishment and maintenance

UL138 significantly but incompletely repressed IE1 transcription during latency, implying that clinical strains may encode an additional IE-suppressive function. TB40/E-ΔUL138 (fig. S2A), a clinical

strain lacking UL138 with replication and gene expression signatures in fibroblasts similar to wild-type TB40/E (fig. S7, A to C), still silenced IE1 transcription in the presence of VPA in CD34⁺ cells (Fig. 2, A and B) and THP-1 cells (Fig. 2, C and D) and IE1 protein accumulation in THP-1 cells (Fig. 2E). This finding indicates that, in addition to Daxx/HDAC and UL138, an additional restriction to IE1 transcription encoded by clinical strain viruses exists. From these data (Fig. 2, A to E), we conclude that UL138 plays a redundant role in the initial silencing of IE1 expression during the establishment of latency (see the model in fig. S8).

In contrast, AD-138HA (Fig. 2F, lane 2) generated as many infectious particles during the latency phase in ESCs as did AD169 (Fig. 2F, lane 1) and far more than did the clinical strain FIX (Fig. 2F, lane 4).

This finding indicates that UL138 is insufficient to maintain latency. From these (Fig. 2F) and previously published data (33), we conclude that UL138 plays a nonredundant role in suppressing infectious progeny generation whereas latency is maintained (see model in fig. S8), because another clinical strain-specific gene is required for latency maintenance (Fig. 2F).

Unlike UL138, this additional clinical strain-specific restriction to IE1 transcription during latency is not located within the ULb' region of the genome. A TB40/E mutant (55) in which the natural ULb' region was deleted and replaced with that of AD169 (TB w/AD-ULb') was still able to silence VPA-induced IE1 transcription in CD34⁺ cells (Fig. 2, G and H) and THP-1 cells (Fig. 2, I and J) and IE1 protein accumulation

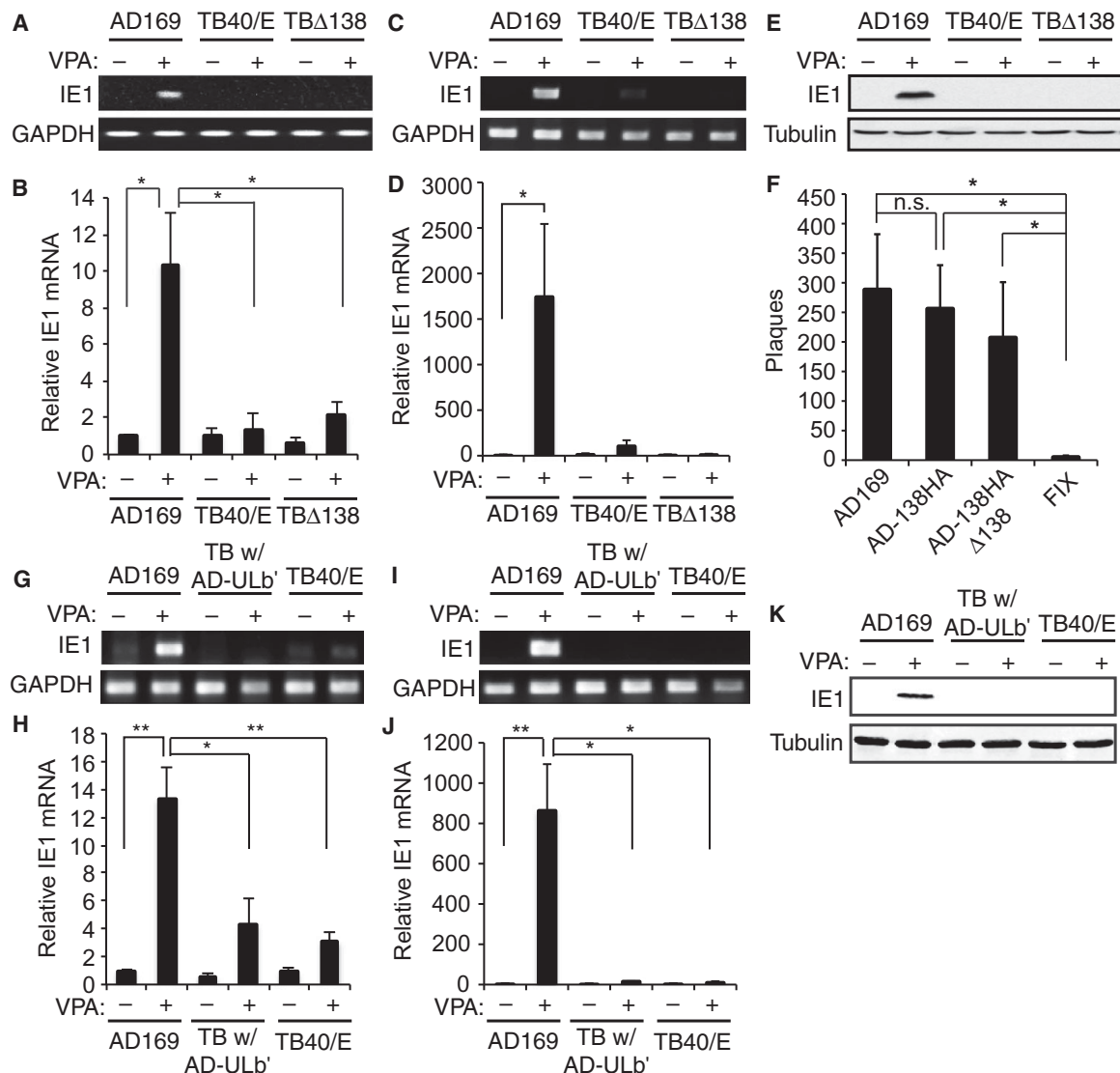


Fig. 2. UL138 is not required for HDAC-independent repression of IE gene expression in clinical strains and is not sufficient to maintain latency. (A to D) CD34⁺ (A and B) or THP-1 (C and D) cells infected with AD169, TB40/E, or TB40/E-ΔUL138 (TB-Δ138) at an MOI of 1 in the absence (-) or presence (+) of VPA were analyzed for the indicated transcripts by RT-PCR (A and C) or qRT-PCR (B and D). (E) Lysates from THP-1 cells infected as in (C) analyzed by Western blot. (F) Infectious virions produced by ESCs infected with the indicated virus at an MOI of 3 for 10 days were quantitated by plaque assay. (G to J) CD34⁺ (G and H) or THP-1 (I and J) cells infected with AD169, TB40/E with ULb' replaced with that of AD169 (TB w/AD-ULb'), or TB40/E at an MOI of 1 in the absence (-) or presence (+) of VPA were analyzed by RT-PCR (G and I) or qRT-PCR (H and J). (K) Lysates from THP-1 cells infected as in (I) analyzed by Western blot. Data are means ± SEM from three independent experiments. *P < 0.05 or **P < 0.01 by Student's *t* test. n.s., not significant (P = 0.29).

in THP-1 cells (Fig. 2K). The only other HCMV gene that, when deleted, shows a similar latency phenotype to a UL138-null virus (increased generation of progeny virions during the latency period) is UL133 (56). However, TB w/AD-ULb' does not encode UL133; hence, this gene cannot be mediating the effect observed here. We conclude that 1 (or more) of the more than 200 genetic loci within the UL, US, or repeated regions of HCMV encodes a silencer of IE1 transcription during latency that is yet to be identified.

UL138 prevents KDMs from removing repressive epigenetic histone modifications and activating viral IE transcription during latency

The HDAC-independent manner in which UL138 acts pointed toward the modulation of histone methylation as a possible mechanism through which it could inhibit HCMV IE1 transcription during latency. Therefore, we analyzed the effect of UL138 on repressive modifications of histones associated with the MIEP. We found that the repressive marks H3K27me3 (Fig. 3A) and H3K9me2 (Fig. 3B) were present at the MIEP in AD-138HA-infected THP-1 cells but decreased significantly in cells infected with wild-type AD169. In CD34⁺ cells, H3K27me3 (Fig. 3C) but not H3K9me2 (Fig. 3D) was enriched at the

MIEP in the presence of UL138. Because the H3K27me3 mark is present at the MIEP of AD169 genomes in THP-1 cells (46) (Fig. 3A) and CD34⁺ cells (Fig. 3C), UL138 cannot be required for its writing. Therefore, we reasoned that the mechanism through which UL138 suppresses HCMV transcription was less likely to result from enhancing the writing of this repressive mark, and more likely from the inhibition of its removal by KDMs.

We found that the H3K27me3 demethylase JMJD3 (KDM6B) (57) was recruited to the AD169 MIEP in THP-1 cells treated with VPA, even when viral transcription was inhibited by UV-inactivating viral genomes (Fig. 3, E and F) or if all RNA polymerase II transcription was inhibited with actinomycin D (Fig. 3, G and H). Thus, JMJD3 recruitment does not appear to be a result of viral transcription. To determine whether UL138 had any effect on KDM recruitment, we used chromatin immunoprecipitation (ChIP) assays to quantitate the association of JMJD3 and the H3K9me2 demethylase LSD1 (KDM1A) with the MIEP during latency. We found that UL138 significantly decreased JMJD3 association with the MIEP in THP-1 cells (Fig. 3I) and CD34⁺ cells (Fig. 3J). Likewise, UL138 significantly decreased LSD1 association with the MIEP in THP-1 cells (Fig. 3K) and CD34⁺ cells (Fig. 3L).

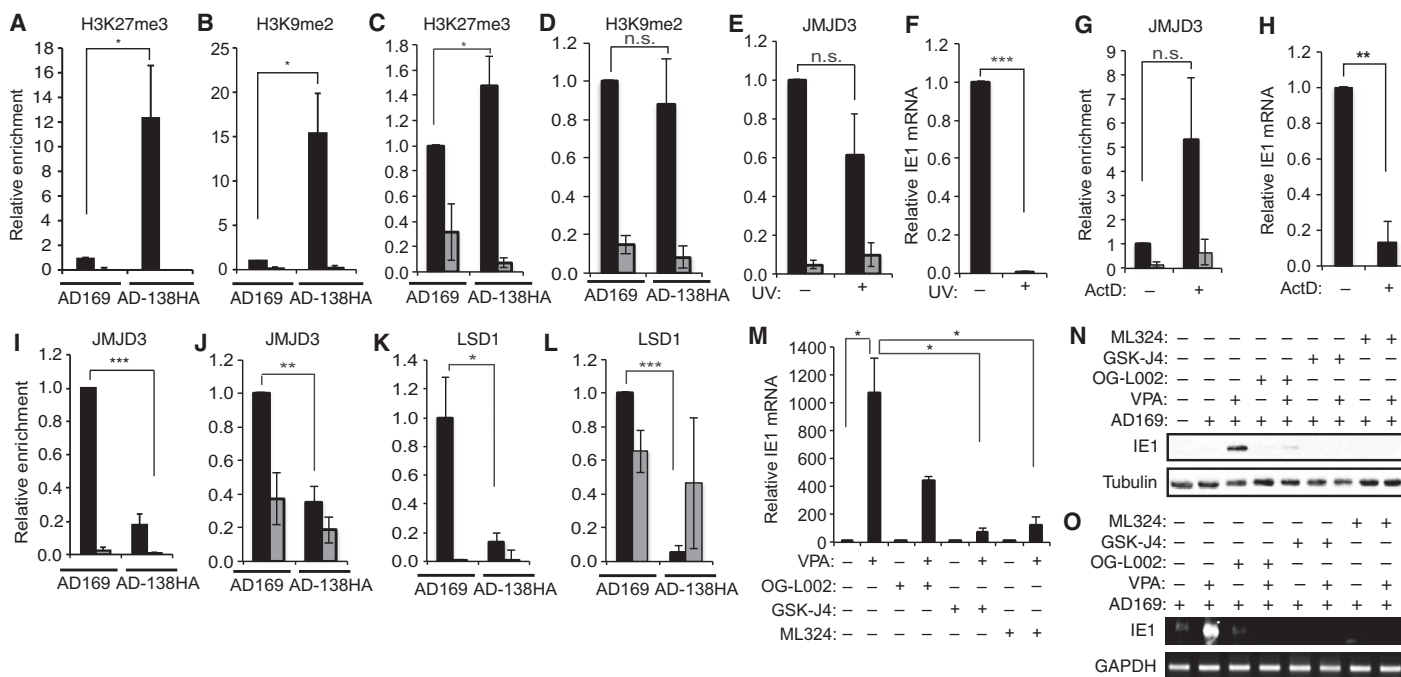


Fig. 3. UL138 inhibits demethylation of histones at the MIEP to facilitate silencing of IE1 expression during latency. (A to D) ChIP assays for repressive histone methylation marks H3K9me2 (A and C) and H3K27me3 (B and D) at the MIEP in VPA-treated THP-1 (A and B) or CD34⁺ (C and D) cells infected with the indicated virus at an MOI of 3 or 1 for 18 or 24 hours, respectively. (E) ChIP assays for the presence of the histone demethylase JMJD3 at the MIEP in VPA-treated THP-1 cells infected with live (–) or UV-inactivated (+) AD169 at an MOI of 1 for 18 hours. (F) THP-1 cells infected as in (E) analyzed for IE1 transcripts by qRT-PCR. (G) ChIP assay for JMJD3 at the MIEP in VPA-treated THP-1 cells infected with AD169 at an MOI of 1 for 6 hours in the absence (–) or presence (+) of actinomycin D (ActD). (H) THP-1 cells infected as in (G) analyzed for IE1 transcripts by qRT-PCR. (I to L) ChIP assays for the presence of JMJD3 (I and J) or LSD1 (K and L) at the MIEP in VPA-treated THP-1 (I and K) or CD34⁺ (J and L) cells infected with the indicated virus at an MOI of 3 or 1 for 18 or 24 hours, respectively. (M and N) THP-1 cells were infected with AD169 at an MOI of 1 in the absence (–) or presence (+) of VPA and the histone demethylase inhibitor OG-L002, GSK-J4, or ML324 for 18 hours and analyzed by qRT-PCR (M) or Western blot (N). (O) CD34⁺ cells were infected with AD169 at an MOI of 1 in the absence (–) or presence (+) of VPA, OG-L002, GSK-J4, or ML324 for 24 hours and analyzed by RT-PCR. In (A) to (E), (G), and (I) to (L), gray bars represent immunoglobulin G (IgG) controls. Data are means ± SEM from at least three independent experiments. **P* ≤ 0.05, ****P* < 0.01, or *****P* < 0.001 by Student's *t* test. n.s., not significant (*P* > 0.1). For all ChIP assays, the signal from a specific antibody was significantly enriched over the IgG signal (*P* < 0.05) with the exception of LSD1 ChIP in AD-138HA-infected CD34⁺ cells (L), which is not significantly different from IgG (*P* = 0.18).

If inhibiting KDM association with the MIEP were the mechanism through which UL138 suppressed IE1 transcription, then KDM inhibitors should phenocopy UL138 expression and inhibit VPA-responsive IE1 expression during AD169 infection. Indeed, we found that GSK-J4, an inhibitor of both JMJD3 and UTX (KDM6A), prevented VPA from activating IE1 mRNA (Fig. 3M) and protein (Fig. 3N) accumulation in THP-1 cells and mRNA accumulation in CD34⁺ cells (Fig. 3O) infected with AD169. Inhibitors of the KDMs that erase repressive H3K9 methylations, ML324 (JMJD2/KDM4A) and OG-L002 (LSD1), also suppressed VPA-responsive IE1 expression (Fig. 3, M to O). From this, we conclude that UL138 silences IE gene expression during latency by preventing the recruitment of KDMs to the MIEP, thereby maintaining repressive histone methylation marks, the removal of which would facilitate transcriptional activation. Whether UL138 also increases the writing of these repressive histone methylation marks (which could also contribute to their maintenance) has not been investigated.

Like UL138, the cellular protein CtBP1 can localize to the Golgi and modifies the functions of KDMs (58, 59). CtBP1 associates with the MIEP in CD34⁺ cells (Fig. 4A) and THP-1 cells (Fig. 4B) latently infected with AD169 but not in those infected with AD-138HA. CtBP1 is a dehydrogenase inhibited by the methionine salvage pathway intermediate MTOB (60, 61). MTOB treatment of THP-1 cells not only inhibited CtBP1 association with the MIEP (Fig. 4C) but also inhibited the ability of VPA to rescue IE1 protein accumulation in THP-1 cells (Fig. 4D) and transcript accumulation in CD34⁺ cells (Fig. 4E). Thus, CtBP1 inhibition by MTOB promotes HCMV latency in a manner similar to UL138 expression. Although the KDM inhibitor ML324 prevents HCMV and HSV-1 IE transcription during lytic infections (62) (Fig. 4, F and G), MTOB failed to inhibit HCMV (Fig. 4F) or HSV-1 (Fig. 4G) IE transcription during productive replication in fibroblasts. These data indicate that CtBP1 appears to be required for the activation of IE1 transcription only during latency in undifferentiated myeloid cells and not during lytic herpesvirus infections in fibroblasts.

CtBP1 and KDMs exist in multiple large complexes and sometimes in the same complex. How CtBP1 targets specific promoters is unclear (58). Likewise, the means through which KDMs are recruited to promoters is not well understood (63) but likely shows cell-type specificity because different KDM-containing complexes form in different cell types (64). How Golgi-localized UL138 antagonizes both CtBP1 (Fig. 4, A and B) and KDM (Fig. 3, I to L) association with the MIEP and whether this happens through direct, indirect, unified, or unique mechanisms remain to be determined (see Fig. 5 and Discussion).

DISCUSSION

HCMV afflicts more neonates than does Down syndrome (65), causes transplant loss, and promotes immunosenescence, cardiovascular diseases (66), and perhaps certain cancers (67). Reactivations of latent infections provide multiple opportunities for the virus to spread and cause disease. HCMV uses multiple mechanisms to keep the levels of its IE proteins low (and perhaps absent) during latent infections. First, it uses a promoter for the expression of its major IE proteins that is more than 1000-fold weaker in the CD34⁺ cells where latency is established than in fibroblasts where lytic infection occurs (20). Second, it prevents pp71-mediated neutralization of the Daxx/HDAC intrinsic cellular defense that silences IE gene expression by keeping the tegument-delivered protein in the cytoplasm (19–21, 51). Third, it allows KAP1-

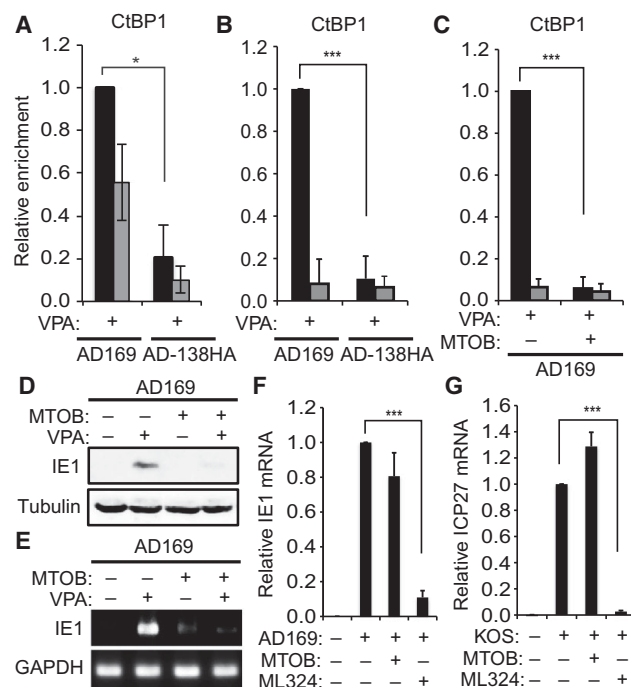


Fig. 4. UL138 prevents recruitment of CtBP1 to the MIEP to silence IE1 transcription during latency. (A and B) ChIP assays for CtBP1 at the MIEP in VPA-treated CD34⁺ (A) or THP-1 (B) cells infected with the indicated virus at an MOI of 1 or 3 for 24 or 18 hours, respectively. (C) ChIP assays for CtBP1 at the MIEP in VPA-treated THP-1 cells infected with AD169 at an MOI of 3 in the absence (–) or presence (+) of the CtBP1 inhibitor MTOB for 18 hours. In (A) to (C), gray bars represent IgG controls. (D and E) THP-1 (D) or CD34⁺ (E) cells were infected with AD169 at an MOI of 1 in the absence (–) or presence (+) of VPA or MTOB and analyzed for IE1 expression by Western blot (D) or RT-PCR (E). (F and G) NHDFs infected with AD169 (F) or HSV-1 KOS (G) at an MOI of 0.1 in the absence (–) or presence (+) of MTOB or ML324 for 6 hours and analyzed for IE1 (F) or ICP27 (G) expression by qRT-PCR. Data are means ± SEM from at least three independent experiments. **P* < 0.05 or ****P* < 0.001 by Student's *t* test. For all ChIP assays, the signal from the CtBP1 antibody was significantly enriched over the IgG signal (*P* < 0.05) with the exception of AD-138HA-infected CD34⁺ cells (A), which is not significantly different from IgG (*P* = 0.2).

mediated transcriptional silencing (26). Fourth, the viral UL138 protein maintains silencing by impairing KDM-mediated activation of IE1 transcription in undifferentiated myeloid cells (Fig. 5). Mechanisms to prevent accumulation of the IE proteins also exist, including decreases in viral IE mRNA stability or translation by cellular (the has-miR-200 family) (68) and perhaps viral (miR-UL112-1) microRNAs (69, 70).

The viral effort extended to suppress IE protein accumulation during latency appears to underscore the importance of achieving this goal for both inhibiting untimely replication and protecting latently infected cells from immune detection and clearance, as IE1 encodes prominent T cell epitopes (71). The initial dependence on cellular proteins such as KAP1, Daxx, and HDACs to silence IE genes during latency may allow this critical step to occur rapidly, perhaps in the absence of de novo viral gene expression. Subsequent repression by viral factors such as UL138 appears to transfer command over viral gene expression from the cell to the virus, which may be important for

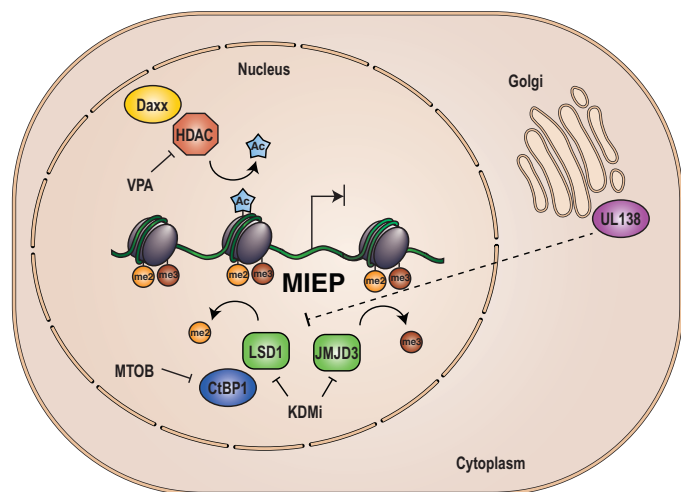


Fig. 5. Model for the regulation of HCMV IE gene expression by cellular intrinsic defenses during the establishment of latency. The Daxx intrinsic defense is active during latency and restricts IE gene expression by recruiting an HDAC to deacetylate histones associated with the MIEP. Artificial inactivation of this defense with the HDAC inhibitor VPA results in activation of IE gene expression. The antilient histone KDM and CtBP1-mediated intrinsic defense facilitates activation of IE gene expression by removing repressive histone methylations from the MIEP. Pharmacological inhibition with KDM inhibitors (KDMi) or the CtBP1 inhibitor MTOB or expression of UL138 prevents KDM-mediated activation of IE gene expression and promotes the establishment of latency.

effectively controlling maintenance and reactivation events. The nonintuitive silencing (Daxx, KAP1), desilencing (KDMs), resilencing (UL138) progression may provide a window for the transcription of a novel protein encoded by IE1 exon 4 that has been proposed as a potential determinant for genome maintenance during latency (72).

UL138 appears to suppress HCMV IE1 transcription from its location at the Golgi apparatus (Fig. 5). Whether it does this directly by sequestering some trans-activating factor or indirectly by regulating a signaling pathway is under investigation. Effects on nuclear transcription instituted by cytoplasmic proteins are well established. Examples include growth factor receptors (for example, epidermal growth factor receptor, platelet-derived growth factor receptor, insulin-like growth factor receptor) (73), innate immune sensors [for example, Toll-like receptors, RIG-I (retinoic acid-inducible gene-I)-like receptors, mitochondrial antiviral signaling protein, stimulator of interferon genes] (74), and components of other cellular signal transduction pathways (75–77). Traditionally, proteins with such activity are encoded by cellular genes. Work here highlights that viruses can encode such proteins as well to regulate their own gene expression.

We describe CtBP1 and KDM delivery to the MIEP as an intrinsic defense against latent HCMV (Fig. 5 and fig. S8) that activates a viral process (IE1 transcription). This restriction protects individual cells and the larger host against latent colonization and has likely emerged recently because the viral antidote UL138 is identifiable only in human, chimpanzee, and rhesus cytomegalovirus (56). Neutralization of this defense by UL138 may come at a fitness cost, because the genomic region encoding UL138 is lost during serial lytic infections *in vitro*. Our description extends the definition of intrinsic immunity be-

yond the previously characterized defenses that protect against productive infection by inhibiting a viral process. Although many cellular proteins that activate viral transcription improve viral replication (78–80), CtBP1 and KDMs are unique because their ability to activate HCMV IE1 transcription in undifferentiated myeloid cells appears detrimental to the virus by preventing latency establishment.

HCMV inactivates the Daxx/HDAC defense to promote lytic infection in differentiated cells but uses it to help establish latency in undifferentiated myeloid cells. The KDM-mediated defense shows a reciprocal relationship. It is inactivated in undifferentiated cells to promote latency establishment and used in fibroblasts to promote IE gene expression and lytic infection (62). The successful nature of HCMV infections in the face of a daunting adaptive, innate, and intrinsic immune system attests to the abilities of the virus to manipulate all aspects of human immune safeguards.

The discovery of a cell-autonomous protection against HCMV latency indicates that our immune system learned long ago what scientists only recently discovered: Latent reservoirs are seeded too rapidly for innate or adaptive immune responses to effectively control. Thus, faster-acting intrinsic defenses may be especially effective against a process that happens quickly, and without generating robust targets of innate and adaptive immune responses. Furthermore, the continual requirement of both UL138 and another clinical strain-specific factor to maintain the latent state indicates that although the latent genome may be invisible to innate and adaptive immune responses, it is readily apparent to intrinsic defenses that are presumably always on guard. Our demonstration here of the battles between cellular intrinsic defenses and viral countermeasures highlights the previously underappreciated cell-autonomous nature of immune protection against lifelong colonization by latency-achieving viruses.

MATERIALS AND METHODS

Construction of recombinant viruses

Recombinant mutant viruses were constructed via two-step recombination as described previously by Tischer *et al.* (81) using BAC clones of HCMV strains TB40/E and AD169. See fig. S2A and Supplementary Materials and Methods for details.

Inhibitors and antibodies

VPA (1 mM; Sigma) dissolved in water was added 3 hours before infection for THP-1 cells, 1 hour before infection for ESCs, or at the time of infection for CD34⁺ cells. Actinomycin D (1 µg/ml; Sigma), OG-L002 (25 µM; Selleck Chemicals), GSK-J4 (30 µM; Sigma), or ML324 (50 µM; Sigma) dissolved in dimethyl sulfoxide was added at the time of infection. MTOB dissolved in media was added 18 hours before infection of NHDFs or at the time of infection for THP-1 and CD34⁺ cells. Details of antibodies used can be found in Supplementary Materials and Methods.

Western blots

Cells were lysed in radioimmunoprecipitation assay buffer plus protease inhibitors or 1% SDS, and equal amounts of protein were analyzed by Western blot with indicated primary antibodies and appropriate infrared dye-conjugated secondary antibodies (LI-COR) as described previously by Albright and Kalejta (51). Additional details can be found in Supplementary Materials and Methods.

RT-PCR and qRT-PCR

For RNA analysis, total RNA was isolated, treated with deoxyribonuclease I, and used for RT-PCR as described previously by Saffert *et al.* (20). For qRT-PCR, total RNA was converted to complementary DNA before analysis by qPCR. Viral gene expression was normalized to cellular β -actin or GAPDH and shown relative to untreated AD169-infected controls. Primer sets and additional details can be found in Supplementary Materials and Methods.

Maintenance assays

Latency maintenance assays were performed as described previously by Penkert and Kalejta (21) with minor modifications. Briefly, human ESCs were infected at an MOI of 3 for 10 days before being cocultured with permissive fibroblasts. After 3 days, cocultures were overlaid with agarose and maintained for 10 days before being fixed and stained with methylene blue to identify plaques.

ChIP assays

ChIP assays were performed as described previously by Winkler and Kalejta (82). Precipitating DNA was analyzed by qPCR and normalized to input levels and shown relative to AD169-infected controls. For histone modifications, output-to-input ratios were normalized to the output-to-input ratios for total histone H3 from the same sample. See Supplementary Materials and Methods for further details.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/1/10/e1501164/DC1>

Materials and Methods

Fig. S1. UL138 represses an MIEP reporter in THP-1 cells.

Fig. S2. Phenotypic characterization of AD-UL138HA.

Fig. S3. UL138HA expressed from recombinant AD169 localizes to the Golgi.

Fig. S4. AD-138HA enters CD34⁺ cells and expresses UL138.

Fig. S5. Expression of viral transcripts during experimental latent infections.

Fig. S6. UL138 is required for the phenotype of AD-138HA.

Fig. S7. Phenotypic characterization of TB40/E-Δ138.

Fig. S8. Model for redundant and nonredundant roles of UL138 in the establishment and maintenance of HCMV latency.

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