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Repression of the major immediate early promoter of human cytomegalovirus allows transcription from an alternate promoter

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Abstract

Following infection, the human cytomegalovirus (HCMV) genome becomes rapidly associated with host histones which can contribute to the regulation of viral gene expression. This can be seen clearly during HCMV latency where silencing of the major immediate early promoter (MIEP), normally responsible for expression of the key lytic proteins IE72 and IE86, is mediated by histone methylation and recruitment of heterochromatin protein 1. Crucially, reversal of these histone modifications coupled with histone acetylation drives viral reactivation which can be blocked with specific histone acetyltransferase inhibitors (HATi). In lytic infection, a role for HATi is less clear despite the well-established enhancement of viral replication observed with histone deacetylase inhibitors. Here we report that a number of different broad-acting HATi have a minor impact on viral infection and replication during lytic infection with the more overt phenotypes observed at lower multiplicities of infection. However, specific analyses of the regulation of major immediate early (MIE) gene expression reveal that the HATi C646, which targets p300/CBP, transiently repressed MIE gene expression via inhibition of the MIEP but by 24 h post-infection MIE gene expression was rescued due to compensatory activation of an alternative IE promoter, ip2. This suggested that silencing of the MIEP promoted alternative ip2 promoter activity in lytic infection and, consistent with this, ip2 transcription is impaired in cells infected with a recombinant HCMV that does not auto-repress the MIEP at late times of infection. Furthermore, inhibition of the histone methyltransferases known to be responsible for auto-repression is similarly inhibitory to ip2 transcription in wild-type infected cells. We also observe that these discrete transcriptional activities of the MIEP and ip2 promoter are also reflected in reactivation; essentially in cells where the MIEP is silenced, ip2 activity is easier to detect at very early times post-reactivation whereas in cells where robust activation of the MIEP is observed ip2 transcription is reduced or delayed. Finally, we observe that inhibition of pathways demonstrated to be important for reactivation of HCMV in dendritic cells, e.g. in response to IL-6, are preferentially important for activation of the MIEP and not the ip2 promoter. Together, these data add to the hypothesis that the existence of multiple promoters within the MIE region of HCMV can drive reactivation in a cell type- and ligand-specific manner and also suggest that inter-dependent regulatory activity between the two promoters exists.

INTRODUCTION

Human cytomegalovirus (HCMV) remains an important pathogen in a number of vulnerable patient groups, in part due to the capacity of HCMV to establish lifelong latent infections of the host which can reactivate to cause disease [1–4]. Consequently, a deeper understanding of the molecular and cellular mechanisms that govern HCMV latency has the potential to underpin new strategies to treat HCMV infection *in vivo*.

A key determinant of HCMV latency and reactivation is the differential regulation of the major immediate early promoter (MIEP) responsible for expression of the MIE gene products, IE72 and IE86, essential for efficient productive infection [2, 5]. The differential regulation of the MIEP in latent and lytic infection is linked to the biology of chromatin [6]. Specifically, in latently

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Abbreviations: crs, *cis* repression sequence; DC, dendritic cell; HAT, histone acetyltransferase; HATi, histone acetyltransferase inhibitor; HCMV, human cytomegalovirus; HDACi, histone deacetylase inhibitor; HFF, human fetal foreskin fibroblast; HMT, histone methyltransferase; HP1, heterochromatin protein 1; hpi, hours post-infection; HSV, herpes simplex virus; ip2, internal promoter 2; MIEP, major immediate early promoter; MoDC, monocyte derived dendritic cell; PMA, phorbol 12-myristate 13 acetate; qRT-PCR, quantitative real-time PCR; SFK, src family kinase.

infected CD34+ cells, and their derivative CD14+ monocytes, the MIEP is bound by histones that are extensively methylated on key lysine residues (e.g. H3-K9) that promote recruitment of heterochromatin protein 1 (HP1) resulting in transcriptional silencing [7]. Importantly this chromatinization is dynamic: cellular differentiation of CD34+ cells or monocytes to myeloid macrophage or dendritic cells (DCs) is concomitant with the initiation of intracellular changes that support substantial histone de-methylation and acetylation (e.g. H4ac and H3-K14ac) at the MIEP resulting in viral MIE gene expression and reactivation [7, 8]. These changes in chromatin structure at the MIEP are driven by the activity of viral factors acting in concert with cellular signalling pathways responding to differentiation or inflammation [9–15]. In our own studies, DC inflammation induced activation of ERK-MAPK and Src Family Kinase (SFK) pathways to induce histone phosphorylation (H3-S10p) and recruitment of histone acetyltransferase (HAT) activity to the MIEP to promote viral reactivation [16–18]. Furthermore, this clear dependence on chromatin-mediated regulation of differential MIE gene expression underpins multiple therapeutic strategies seeking to employ diverse epigenetic modifiers to 'purge' latent infections *in vivo* [19, 20].

The identification of alternative promoters within the MIE region [21] has challenged this prevailing view of chromatin-mediated regulation of the MIEP being the major determinant for HCMV reactivation. Instead, it is hypothesized that internal promoters (ip1 and ip2) downstream of the MIEP located within intron A (between exon 1 and exon 2 of the MIE genes) may support viral reactivation in certain models of HCMV latency [22, 23]. However, our own studies suggest that both the MIEP and ip2 promoters are active [24] and this is clearly dependent on cell type and the nature of the reactivation stimulus [24, 25]. From our own studies it is also becoming evident that the sensitivity of the canonical MIEP to epigenetic modifiers is different than that observed for the ip2 promoter – particularly, with p300/CBP inhibitor C646 which appeared to have little impact on ip2 promoter activity [24]. The reasons for this are unclear but the fact that CREB is important for reactivation in DCs [16] may be linked to this cell type's specific sensitivity to p300/CBP activity which modulates the function of CREB. Indeed, multiple studies point towards a role for CREB response elements in the MIEP having very context-specific roles in reactivation [16, 26–29].

The precise role of chromatin in the regulation of viral gene expression during lytic infection is less well understood although clear parallels exist [30]. The classical temporal herpes virus gene expression has been suggested to involve sequential histone modifications on multiple promoters during the course of infection [31–33] and histone deacetylase inhibitors (HDACi) enhance viral gene expression and replication particularly at low m.o.i. [33–35]. Furthermore, IE72 has been shown to sequester HDACs to activate Early (E) and Late (L) viral gene expression and, consistent with this, HDACi also rescue the replication defect of an IE72 deletion virus at similarly low m.o.i. [36, 37]. Furthermore, we reported that infection of permissive human fetal foreskin fibroblasts (HFFs) at low m.o.i. results in the accumulation of histones with ostensibly repressive modifications onto the MIEP during the very early stages of infection [33]. This observation provided an explanation for the pro-viral role of HDACi through the much quicker reversal of this potential silencing. The wealth of data on HDACi and the regulation of viral gene expression suggest that other epigenetic modifiers will play a role in the regulation of the MIEP. In herpes simplex virus (HSV) infection the interaction of tegument protein VP16 with host cell factor 1 results in the recruitment of acetyltransferase activity, which is a key event in the initiation of lytic gene expression [38] and, furthermore, inhibition of histone demethylase activity is sufficient to reduce both HSV and HCMV infection [39, 40]. Finally, silencing of the MIEP at late times of lytic infection by IE86 is also mediated by an interaction with chromatin-modifying enzymes. IE86 binds to the MIEP via the *cis* repression sequence (crs) recruiting HDACs and HMTs to the MIEP in the process to promote extensive histone methylation (H3-K9me) at the MIEP at late times of infection [41, 42].

HATs are a diverse of group of cellular enzymes that can acetylate histone and non-histone proteins and, consequently, are often referred to as lysine acetyltransferases or KATs [43, 44]. Type 1 HATs are located in the cytoplasm and thus type 2 HATs are considered the epigenetic modifiers active in the nucleus. Type 2 HATs are further sub-divided into the p300/CBP (KAT3B and 3A, respectively), GNAT family (Gcn5 and PCAF; KAT2A and 2B), and the larger MYST family comprising Tip60 (KAT5), MOZ (KAT6A), MORF (KAT6B), HBO1 (KAT7) and MOF (KAT8). Consistent with this, they encode bromodomains which promote binding to histones, and the p300/CBP and MYST family members also possess direct DNA binding capacity through zinc finger domains [44]. In the context of HCMV lytic infection, the role of specific HATs is less well understood although the MYST family member Tip60 has been reported to be important for HSV, Epstein–Barr virus (EBV) and HCMV replication during lytic infection [45]. Furthermore, binding of IE86 to PCAF may be important for transactivation of E and L viral gene expression [46]. Thus, we initially sought to better understand the role of HATs in lytic infection.

Here, we report on the outcome of an analysis of HATi activity against lytic infection and observe that at high m.o.i. there is little impact but we do observe some antiviral activity at low m.o.i. in viral spread assays using inhibitors of p300/CBP or Tip60. These differences in replication were correlated with small but reproducible effects on viral E and L gene expression. Furthermore, we also observe that transcription from the canonical MIEP is reduced in the presence of the p300/CBP inhibitor C646, but this is compensated for by the induction of transcription from the alternate ip2 promoter downstream of the MIEP. Interestingly, we also observe that the established transcription from ip2 at early/late times of infection [24–72 h post-infection (hpi)] is reduced in cells infected with a crs-deleted HCMV that does not silence MIEP transcription at later times of infection. Together, these data suggest that the MIEP and ip2 promoter have an interdependent relationship during lytic infection. Pertinently, we also observe the same transcriptional relationship in primary cell models of HCMV latency and reactivation. Specifically, we observe transcription from

ip2 but not the MIEP in partially differentiated DCs and that this is enhanced following phorbol 12-myristate 13-acetate (PMA), but not IL-6, stimulation. In contrast, IL-6 stimulation of immature DCs results in predominantly MIEP-derived transcripts and a concomitant reduction in ip2 transcription at immediate times post-IL-6 stimulation. Finally, we report that inhibition of ERK and SFK signalling disproportionately represses MIEP, but not ip2 promoter activity, in DCs. These data argue that the MIEP is an important determinant of latency and reactivation but that the MIEP and ip2 promoters have a complex interdependent relationship controlling activity. They also suggest that ip2 activity may provide a mechanism for rapid IE gene expression in situations where the MIEP is silenced or where there is a failure to reverse MIEP silencing.

METHODS

Cell culture

HFFs (SCRC-1041) and adult retinal pigment epithelial 19 cells (ARPE-19, CRL-2302) were purchased from ATCC. All cells were grown in high-glucose Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% FBS (ThermoFisher), 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin. For specific experiments HFFs were seeded at 80% density 24 h pre-infection on 96- or 24-well coated plates (Corning).

For single round infection assays, HFFs were infected at an m.o.i.=1 and then samples of supernatants taken at times stated post-infection and analysed by $TCID_{50}$ to quantify virus titres. For viral spread assays, cells were infected at an m.o.i.=0.05 and then left for 10 days with a media refresh at day 5 (including inhibitors where appropriate). After 10 days cells were fixed, stained and quantified as described for indirect immunofluorescence staining (see below).

Cell viability was assayed using Cell Titer-Glo (Promega) as described by the manufacturer. Briefly, 8×10^3 cells were plated in 96-well plates and cultured overnight. Then cells were treated with DMSO control or specific HATi, in combination or JMJD2 inhibitor for 24 h and then analysed for ATP production in a luminometer.

Viruses and inhibitors

The HCMV isolate TB40/e was purified from infected human adult retinal pigment epithelial-19 (ARPE-19) cells then amplified for one round in human fibroblasts and supernatants purified using sorbitol gradients as previously described. Purified virus was then characterized by $TCID_{50}$ on ARPE-19 and HFFs.

The ΔCRS virus has been described previously [41] and was engineered to have a deletion of the crs in the MIEP. The virus was made in a Towne background and both ΔCRS Towne virus and the Towne parent were propagated in HFs as described above.

For inhibition of different HATs the following inhibitors were used: NU9056 (Tocris): aselective Tip60 inhibitor used at 10 µM; MG149 (Axon Medchem): a potent and selective inhibitor of the MYST family of HAT proteins that targets the acetyl-CoA binding site used at 75 µM; CPTH2 (Sigma Aldrich): a selective inhibitor of the Gcn5 network of HAT proteins through a bridge-interaction with histone H3 [47] used at 200 µM; and C646 (Sigma Aldrich): aselective competitive inhibitor of p300/CBP acetyltransferase activity used at 25 µM. Inhibition of histone deacetylase activity was achieved using trichostatin A (330 nM; Sigma). Inhibition of JMJD2 histone demethylase was achieved using ML324 (Sigma; 2.5 µM). Chaetocin (Tocris; 5 µM) and BX01294 (Tocris; 10 µM) were used to inhibit SUV39H1 and G9a histone methyltransferases. Pathway inhibition was achieved using an ERK inhibitor, U0126 (10 µM; Calbiochem) or SFK inhibitor, PP2 (10 µM; Calbiochem).

All compounds were dissolved in DMSO which was used as a solvent control at the appropriate dilution for each inhibitor.

Latency and reactivation experiments

CD14+ monocytes were isolated from venous blood donations from healthy volunteers using Ficoll separation and MACS CD14+ positive cell separation (Miltenyi Biotec) before seeding on plastic and subsequent feeding with X-VIVO-15 supplemented with 2 mM L-glutamine. After 24 h, cells were infected with HCMV TB40/e at an m.o.i. equivalent to 5 on HFFs (routinely about m.o.i.=0.5 on ARPE-19 cells). At 3 days post-infection cells were differentiated with GM-CSF/IL-4 (both 1000 U ml^{-1}; Peprotech) for 4–6 days and a further 3–24 h with PMA (20 nM; Sigma) or IL-6 (500 ng ml^{-1}; Peprotech) to promote reactivation.

Nucleic acid isolation and analysis

To isolate RNA, cells were washed once with PBS before direct harvest of samples with RLT buffer (Qiagen). RNA extraction was then performed as per the manufacturer's instructions using the RNeasy Mini Kit (Qiagen). RNA (or RNA from historical studies) was then amplified by Sybr green (Thermofisher) quantitative real-time (qRT)-PCR using previously published gene-specific primers [13, 22]: UL122-3 (exon 2 and exon 3) 5′-GCGCCAGTGAATTTCTCTTC and 5′-ACGAGAACCCCGAGAAAGATG 3′; MIEP derived IE 5′-TTGACCTCCATAGAAGACAC-3′ and 5′-AGGACTCCATCGTGTCAAGG-3′; ip2 derived (UTR70) 5′-TAGCTGACAGACTAACAGAC-3′ and 5′-AGGACTCCATCGTGTCAAGG-3′; 18S 5′-GTAACCCGTTGAACCCCA-3′ and 5′-CCATCCAATCGGTAGCG-3′; UL44 5′-GTACAACAGCGTGTCGTGCT-3′ and 5′-ATAACCGCGTCAGTTTCCAC-3′; UL138

5′-GAGCTGTACGGGGAGTACGA-3′ and 5′-AGCTGCACTGGGAAGACACT-3′; UL100 5′-CTTTTTCTTCTCGCGTCTGC-3′ and 5′-ACCACGAAGACGGCTAACAC-3′.

Where possible relative expression was analysed using the 2delta delta Ct method comparing controls with test samples. Alternatively to express absolute values in the qPCR analyses, 2delta Ct was used to represent signal above background signal in qPCR.

Western blotting and indirect immunofluorescence staining for viral gene expression

Cells were fixed by treatment with 100% ice-cold ethanol for >20 min at −20 °C. To visualize infection cells were then washed in PBS and incubated with mouse anti-IE (MAB8131; Merck Millipore; 1:2000 dilution) that recognizes both IE72 and IE86 for 1 h, followed by incubation with goat anti-mouse IgG-Alexa-fluor-594nm (Life Technologies; 1:2000 dilution) plus 0.5 µg ml^{-1} DAPI for 1 h. Infection was quantified using Hermes WiScan technology and quantified using Metamorph software.

For Western blotting cells were collected by scraping from the base of the wells and, following centrifugation (600 ***g*** for 5 min), cells were lysed using a 6× protein lysis buffer [60% v/v 1 M Tris (pH 6.8), 120 mg ml^{-1} SDS and 93 mg ml^{-1} DTT with added bromophenol blue; the buffer was diluted with ddH_2O immediately before use and 1:200 benzonase was added]. Ten microlitres of sample was loaded into wells on pre-cast 4–16% gels (Bio-Rad). Following electrophoresis, the proteins were transferred to nitrocellulose membranes (Thermo Scientific). The membranes were blocked for 1 h at room temperature in 4% milk (Marvel) TBS-T [137 mM NaCl, 20 mM Tris-HCl (pH 7.6), 0.5% Tween 20]. The membranes were incubated with primary antibody (diluted in 4% milk TBS-T) overnight at 4 °C before being washed with TBS-T and incubated with secondary antibody (diluted in 4% milk TBS-T) for 1 h at room temperature. The membranes were washed with TBS-T before application of chemiluminescent substrate (Thermo Scientific SuperSignal). Antibodies to the following proteins were used: mouse anti-HCMV IE (1:1000; MAB8131; EMD Millipore), rabbit anti-actin (1:1000; Cell Signaling), goat anti-mouse IgG-HRP (1:10 000; sc-2005; Santa Cruz Biotechnology), and goat anti-rabbit IgG-HRP (1:10 000).

Statistical analyses

A one-way ANOVA was applied to test for variance within the mean and then either a Tukey's or Dunnett's comparison was performed to identify specific means with statistically significant differences. Throughout a non-parametric distribution was assumed. Statistical analyses were only applied if $n>2$. All scatter plots and bar charts depict the mean and one standard deviation from the mean. Significance was assumed if $P<0.05$.

RESULTS

HAT inhibitors limit HCMV infection in an m.o.i.-dependent manner

Epigenetic modifiers that reverse chromatin-mediated silencing of gene expression (e.g. HDACi and BETi) are well documented for their role in the regulation of gene expression during lytic and latent HCMV infection. Specifically, during HCMV lytic infection HDACi have been demonstrated to enhance MIE gene expression and viral replication particularly at low m.o.i. [33, 36]. Having recently established that certain HATi could limit MIE gene expression upon reactivation in DCs [18, 24], we tested whether the same HATi also had any impact on lytic infection. First, we analysed whether HATi had any impact on IE RNA expression at early times post-infection. HFFs pre-treated with HATi were infected (m.o.i.=1) and RNA was harvested at 8 hpi. The data show that pre-treatment with p300/CBP inhibitor C646 resulted in a reduction in IE mRNA expression (Fig. 1a). In contrast, smaller effects were observed with a general MYST family inhibitor, MG149, specific Tip60 inhibitor NU9056 or GNAT/Gcn2 family inhibitor CPTH2 (Fig. 1a). Next HFFs, pre-treated with a specific HATi, or a combination for 3 h, were infected with HCMV and then analysed by immunofluorescence for IE expression 24 hpi. This, however, showed that HATi individually had no impact on infection (Fig. 1b) and, even when used in combination, had only a minor effect (Fig. 1c). In contrast, an inhibitor of histone demethylase, JMJD2, clearly blocked infection (Fig. 1b, c). Consistent with HATi having no major impact on the number of IE-positive cells by immunofluorescence, the inhibitors similarly had no major impact on IE72 levels when analysed by Western blot (Fig. 1d). Importantly, during the timeframe of these analyses none of the inhibitors used had an appreciable impact on cell viability (Fig. 1e). A lack of effect on IE gene expression was also reflected by minimal impact on expression of an early gene, UL44 (Fig. 1f), or the replication of the virus in a single-round replication assay (Fig. 1g). However, an analysis of Gcn5 inhibitor CPTH2 was not possible as it promoted substantial cell death in virally infected cells under long-term culture which precluded it from the analyses on viral replication.

Accumulating evidence suggests that the enhancement of lytic replication by HDACi is most evident at low m.o.i. Similarly, the growth defect of the IE72 deletion virus observed at low m.o.i. is rescued by HDACi [36]. Thus, we next tested whether HATi had a clearer phenotype in low m.o.i. infections. HFFs, pre-treated with HATi inhibitors, were infected with HCMV and then RNA was harvested 24 hpi (Fig. 2a). The data show that no difference in IE gene expression was observed at 24 hpi between control and inhibitor-treated cells (Fig. 2a). Next, we assessed whether the inhibitors had any impact on the growth of the virus at low m.o.i. To do this, cells infected with HCMV (m.o.i.=0.01) were then cultured with inhibitor at 24 hpi and then infection was assessed by immunofluorescence at 10 days. The data suggested that treatment with HATi impacted the spread of HCMV with

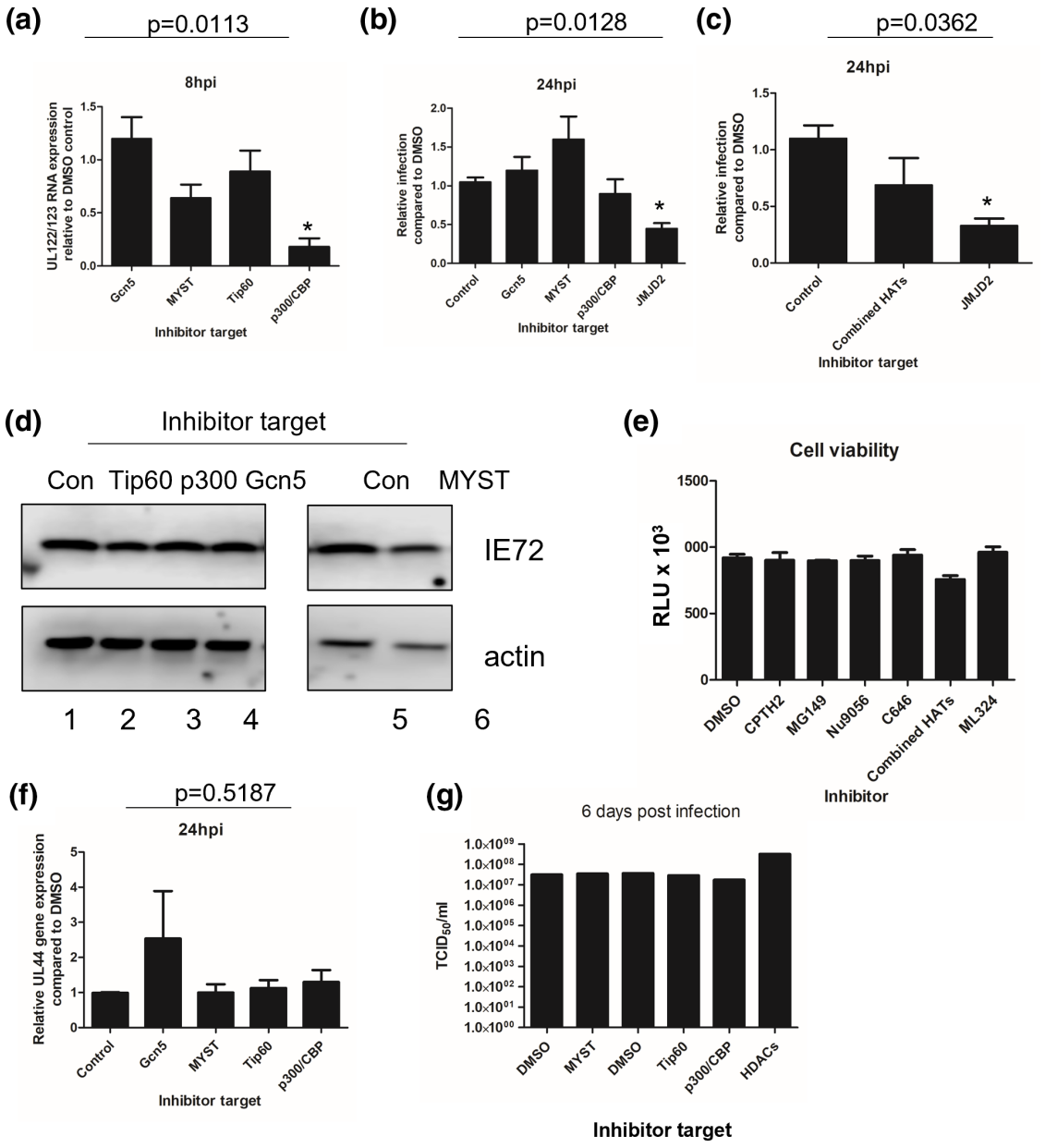

Fig. 1. HATi have a minor impact on HCMV infection at high m.o.i. (a) HFFs were pre-treated with HATi CPTH2 (Gcn5), MG149 (MYST), Nu9056 (Tip60) or C646 (p300/CBP) for 3 h and then infected with HCMV. At 8 h RNA was isolated, converted to cDNA and analysed by qPCR for IE (UL122/UL123) and 18S rRNA expression. IE expression was expressed relative to an equivalent DMSO control. One-way ANOVA with Tukey's multiple comparison of means test to compare means was performed. ***P*<0.01. (b,c) HFFs pre-treated with media (control), CPTH2 (Gcn5), MG149 (MYST), C646 (p300/CBP) or ML324 (JMJD2) or in combination (combined HATs) and then stained 24 hpi for IE expression and infected cells were quantified by Hermes automated scanning. Infection was then expressed relative to the specific DMSO control. One-way ANOVA with Dunnett's correction to compare means was performed. **P*<0.05. (d) HFFs pre-treated with DMSO (con), CPTH2 (Gcn5), MG149 (MYST), C646 (p300/CBP) or Nu9056 (Tip60) were infected and then analysed by Western blot for IE and actin protein levels at 24 hpi. Representative of *n*=3. (e) Cells were incubated with individual HATi inhibitors, combined HATi or ML324 (JMJD2 inhibitor) used in the studies and assayed for viability using Cell Titre Glo (*n*=2). (f) HFFs were pre-treated with media (control) CPTH2 (Gcn5), MG149 (MYST), Nu9056 (Tip60) or C646 (p300/CBP) for 3 h and then infected with HCMV. At 24 h RNA was isolated, converted to cDNA and analysed by qPCR for IE (UL44) and 18S rRNA expression. UL44 expression was expressed relative to equivalent DMSO control and subject to one-way ANOVA. (g) HFFs were infected with HCMV (m.o.i.=5) and then incubated with DMSO, MG149 (MYST), Nu9056 (Tip60), C646 (p300/CBP) or TSA and viral replication was assayed (*n*=2) at 5 days post-infection by $TCID_{50}$. Unless otherwise stated, *n*=3 for all experiments. Error bars represent ±1 SD from the mean.

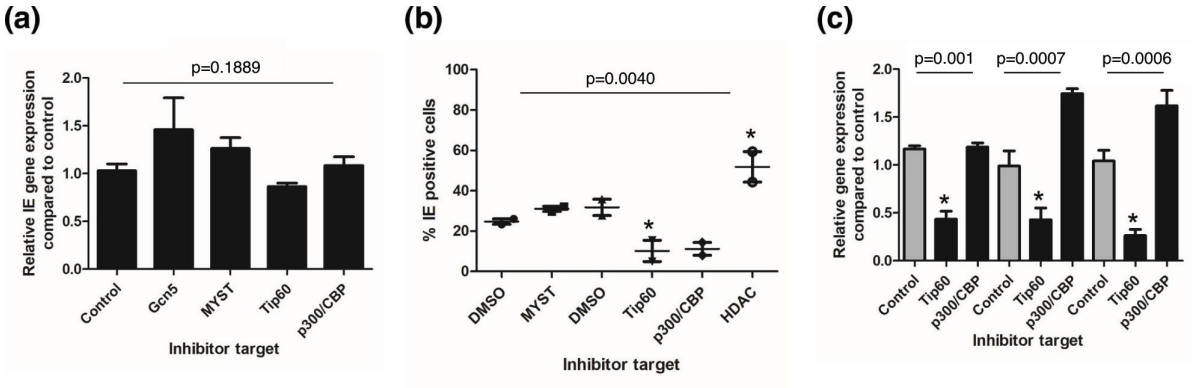

Fig. 2. HATi have more effect on infection at low m.o.i. (a) HFFs were pre-treated with HATi CPTH2 (Gcn5), MG149 (MYST), Nu9056 (Tip60) or C646 (p300/CBP) for 3 h and then infected with HCMV. At 24 h RNA was isolated, converted to cDNA and analysed by qPCR for total IE and 18S rRNA expression. Total IE expression was expressed relative to an equivalent DMSO control. (b) HFFs were infected with HCMV (m.o.i.=0.01) and then incubated with DMSO, MG149 (MYST), Nu9056 (Tip60), C646 (p300/CBP) or TSA (HDAC) at 24 hpi and viral replication and spread were assayed at 10 days post-infection by staining for IE expression and infected cells were quantified by Hermes automated scanning. Infection was then expressed relative to a specific DMSO control. One-way ANOVA coupled with Dunnett's correction to compare means was performed. *P<0.05. (c) HFFs infected with HCMV (m.o.i.=0.05), DMSO, MG149 (MYST), Nu9056 (Tip60), C646 (p300/CBP) or TSA (HDAC) at 24 hpi were analysed for UL54, UL138 and UL100 gene expression by qPCR with expression expressed relative to an equivalent DMSO control. Unless otherwise stated, n=3 for all experiments. Error bars represent ±1 SD from the mean.

evidence of the C646 (p300/CBP) and Nu9056 (Tip60) inhibitors reducing the spread of HCMV in cells. However, only Tip60 inhibition along with the increased spread induced by the HDACi TSA reached statistical significance by ANOVA (Fig. 2b). We also observed that inhibition of Tip60 resulted in reduced expression of multiple post-IE viral gene expression at 48 hpi (Fig. 2c) although, intriguingly, C646 had no major impact on post-IE gene expression, suggesting potentially different targets or mechanisms of action. Overall, the data did suggest that some HATi could impact on viral replication at lower m.o.i. but these effects only reached statistical significance for Tip60 inhibition – especially when compared to the well-established effects of HDACi.

Alternative IE promoters rescue IE gene expression in the context of MIEP inhibition

A lack of any major phenotypes with the HATi along with the risk of toxicity associated with using these inhibitors in long-term cultures led us to change focus and investigate the incongruent effects of HATi on IE gene expression observed 8 hpi (Fig. 1a) compared to 24 hpi (Fig. 2a). Focusing on C646, we confirmed that any impact on IE gene expression observed was transient as, by 24 hpi, there was no difference between control and C646-treated cells at both low and high m.o.i. (Fig. 3a). The identification of alternative promoters that drive MIE gene expression during lytic infection also led us to speculate on the identity of the IE transcripts. Utilizing a qPCR approach that identifies the origin of the MIE transcripts [22, 24], we observed that, consistent with a previous study, transcription from both the MIEP and ip2 promoter was evident at 16 and 24 hpi during lytic infection (Fig. 3b, c). Furthermore, we noted in cells treated with C646 that MIEP transcription was lower but, concomitantly, ip2 transcription was higher when compared to control cells (Fig. 3b, c). These data suggested that the alternative ip2 promoter partially compensated for the loss of MIEP-derived transcription in response to a HATi. Thus, the ip2-derived transcripts provide an alternative template for IE protein translation, giving a possible explanation for the no changes seen IE protein levels observed in Fig. 1. Furthermore, the C646 HATi data also suggested that the ip2 promoter was not inhibited by C646 – an observation that could also be inferred in THP1 models of HCMV reactivation where ip2 transcription is observed even in the presence of C646 [24].

Based on these and previous data we considered the possibility that earlier induction of ip2-derived MIE transcription, in cells where the MIEP was less active, could potentially reflect an inter-dependent relationship between the activity of the two promoters, and we considered ways to investigate this. The presence of a crs within the MIEP allows its targeting by IE86 to promote inhibition of the MIEP at post-IE times of infection (auto-repression [41, 42]). We hypothesized that this silencing of the MIEP may also be a factor in the activation of the ip2 promoter observed later in infection in a previous study [21]. Thus, we infected cells with either wild-type or the ΔCRS virus (a virus in which the crs has been mutated to prevent IE86 binding) and analysed RNA across the course of the infection by qPCR for MIEP- and ip2-derived transcripts (Fig. 4a, b) and established the ratio between the qPCR signals of the two transcripts expressed over time. At 6 hpi the relative values of MIEP- and ip2-derived transcripts in the qPCR analyses (with approximately 10 times greater signal in the MIEP qPCR) was similar for both viruses (compare Fig. 4a, b). Consistent with the concept that as infection proceeds MIEP activity decreases but ip2 activity increases, the relative ratio skewed towards ip2 transcripts which was evident up to 96 hpi in wild-type infection (Fig. 4a). In contrast, the same trend was

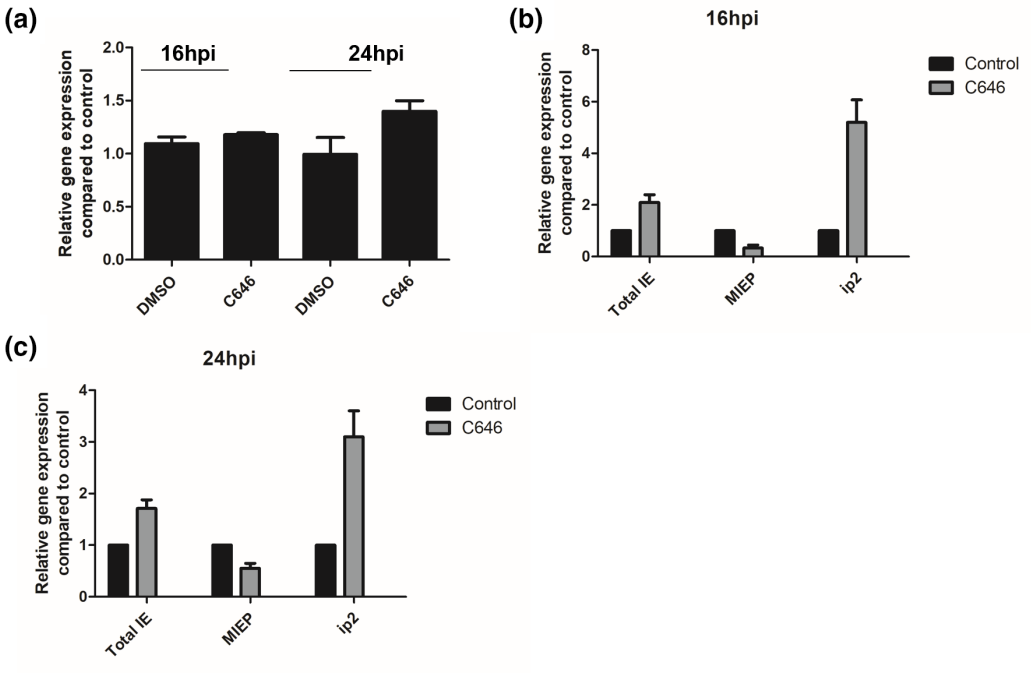

Fig. 3. Transcription from the ip2 promoter is enhanced in C646-treated cells. HFFs were pre-treated with p300/CBP inhibitor C646 for 3 h then infected with HCMV at an m.o.i.=0.1. At 16 and 24 h RNA was isolated, converted to cDNA and analysed by qPCR for 18S expression as a control. (a–c) IE (a), or differential expression of MIEP-derived (MIEPd) and ip2-derived (UTR70) transcripts (b,c). Viral gene expression was expressed relative to an equivalent DMSO control. Unless otherwise stated, *n*=3 for all experiments. Error bars represent ±1 SD from the mean.

not observed in the ΔCRS virus-infected cells where the ratio between the two transcripts did not show the same trend (Fig. 4b) consistent with the lack of MIEP silencing seen in this virus.

We complemented these genetic studies with pharmacological experiments which relied on the understanding that IE86-mediated silencing through the crs is, at least in part, via the recruitment of histone methyl-transferase (HMT) activity to the MIEP – specifically, G9a and SUV39H1 [41]. Thus, we next tested whether inhibition of HMT activity in wild-type infection had the same effect. HFFs infected with HCMV were incubated at 6 hpi with Chaectocin – an inhibitor of both SUV39H1 and G9a as well as the G9a-specific inhibitor, BIX01294, and then MIEP and ip2 transcript levels were measured at 24 hpi. The toxicity of prolonged exposure of the cells to the HMT inhibitors precluded an analysis at later time points. However, even at 24 hpi, it was clearly evident that HMT inhibitors changed the transcriptional profile with reduced expression of ip2 (Fig. 4c) concomitant with elevated MIEP-derived transcription in the presence of HMT inhibitors (Fig. 4d).

MIEP and ip2 promoter activity is inversely correlated in latent infection and reactivating DCs in response to IL-6

Deletion of the internal ip2 promoter has been demonstrated to be non-essential for lytic replication in HFFs but has an HCMV reactivation phenotype in a cell type-dependent manner [22, 24]. In monocyte-derived dendritic cells (MoDCs) stimulated to reactivate with IL-6, we have observed that MIEP transcription predominates. In contrast, in the THP1 cell line, phorbol ester (PMA) stimulation promotes both MIEP- and ip2-driven transcription [24]. In a previous study, investigators showed that low-level ip2 transcription was detectable in latently infected THP1 and CD34+ haematopoietic progenitor cell cultures [22] and, thus, we investigated whether this could also be true in monocytes at different stages of their differentiation to DCs. CD14+ monocytes were cultured with IL-4/GM-CSF and then RNA harvested at 4 days (pre-DC) and 6 days (standard MoDC) later, alongside equivalent cells stimulated with IL-6 or PMA for a further 3–24 h (Fig. 5). The data show that ip2 transcripts are more abundant in day 4 differentiated but unstimulated pre-DCs when compared to standard 6 day differentiated immature DCs (Fig. 5a) – indeed, ip2 qPCR values in 6 day differentiated immature DCs were rarely detected above baseline control. In contrast, MIEP transcription was rarely detected above baseline in equivalent samples, consistent with previous observations using these primer sets. Next, we assessed the impact of adding IL-6 to 4 day and 6 day differentiated DCs on the levels of MIEP- or ip2-derived transcripts and observed that IL-6 induced detectable MIEP-derived transcripts by 3 h post-IL-6 treatment and that this was more robust in day 6 MoDCs although donor to donor variation was evident (Fig. 5b, c). We also observed that IL-6 stimulation did result in ip2 transcription (Fig. 5d, e) but was detectable much earlier in the 4 day DCs compared to 6 day DCs

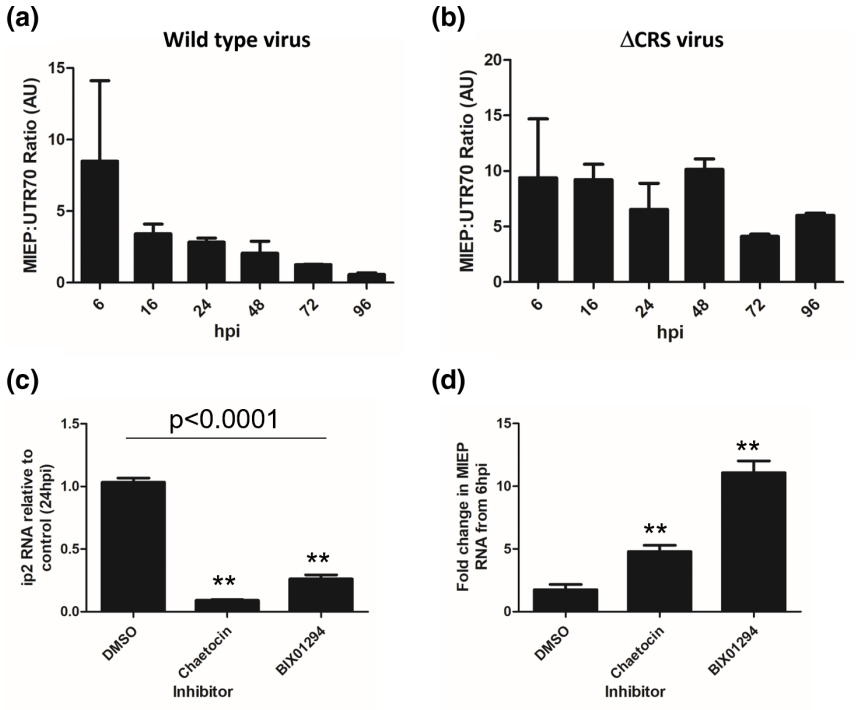

Fig. 4. ip2 transcription is reduced when MIEP auto-repression is inhibited. (a,b) HFFs were infected with Towne (a) or Towne ΔCRS (b) at an m.o.i.=1 and then RNA was harvested between 6 and 96 hpi and analysed for MIEP- and ip2-derived transcripts. Relative expression was expressed as the ratio of MIEP versus ip2 transcripts (AU); *n*=2. (c,d) HFFs were infected with HCMV and then incubated with chaetocin (G9a and SuVar) or BIX01294 (G9a) inhibitor or DMSO control and analysed for ip2 (c) or MIEP (d) expression at 24 hpi. For MIEP, the change in transcription from 6 hpi is shown but no ip2 transcription was detected at 6 hpi. One-way ANOVA with Dunnett's correction was used to identify differences in the means, asterisks denoting statistically significant differences (**P<0.01). Unless otherwise stated, *n*=3 for all experiments. Error bars represent ±1 SD from the mean.

(Fig. 5d, e). Finally, by 24 h of IL-6 treatment, it was evident that 6 day DCs supported robust MIEP transcription in response to IL-6 (Fig. 5c). Next, we analysed the transcriptional profile of the same donor cells but stimulated with PMA. In contrast to IL-6, PMA stimulation of 4 day differentiated DCs promoted both MIEP and ip2 transcription although the induction of ip2 transcripts was much higher (Fig. 5f, h). Similarly, in 6 day differentiated DCs PMA induced both ip2- and MIEP-derived transcripts, and although this was more variable between donors, there was an accumulation of both transcripts between 3 and 24 h post-PMA (Fig. 5g, i). Finally, we analysed the composite data from the three donors over two time points and assessed the impact of IL-6 and PMA on MIEP- and ip2-driven transcription. This composite analysis suggested that IL-6 was more efficient at promoting MIEP transcription in 6 day differentiated DCs compared to 4 day differentiated DCs whereas ip2-derived transcript levels were higher in 4 day differentiated DCs in response to IL-6 (Fig. 5j). In contrast, PMA clearly activated the MIEP equally well in both 4 and 6 day differentiated DCs but also was particularly potent at activating ip2 in the immature 4 day differentiated DCs (Fig. 5k).

ERK and SFK inhibitors block MIEP transcriptional activity in response to IL-6

The differential transcriptional activity of the MIE region in the 4 day differentiated DCs provided a potential tool to investigate whether pathways that we have previously identified as being important for reactivation of HCMV in DCs in response to IL-6 [13, 18] had differential effects on the two viral promoters. Infected monocytes were differentiated for 4 days (with IL-4/GM-CSF). These latently infected cells were then treated with ERK or SFK inhibitors (which we have previously observed to block HCMV reactivation in 6 day differentiated DCs) prior to IL-6 and PMA treatment to assess the impact of ERK or SFK inhibitors on IL-6/PMA induction of MIEP- and ip2-driven transcription (Fig. 6). Interestingly, the addition of ERK inhibitor U0126 decreased MIEP transcription in response to both IL-6 (Fig. 6a) and PMA (Fig. 6b). However, we noted that the reduction in MIEP transcription was concomitant with an increase in ip2 promoter activity in response to both stimuli (Fig. 6a, b). Finally, we next analysed the SFK inhibitor, PP2, for its effect on MIEP- and ip2-driven transcription. Although the phenotypes were smaller, we did observe that, consistent with the ERK inhibitor data, PP2 also reduced MIEP transcription in response to IL-6 (Fig. 6c) and increased ip2-derived expression (Fig. 6c). However, PP2 had no inhibitory impact on PMA-induced activation of the MIEP or ip2 where instead a small increase in MIEP activity was observed (Fig. 6c).

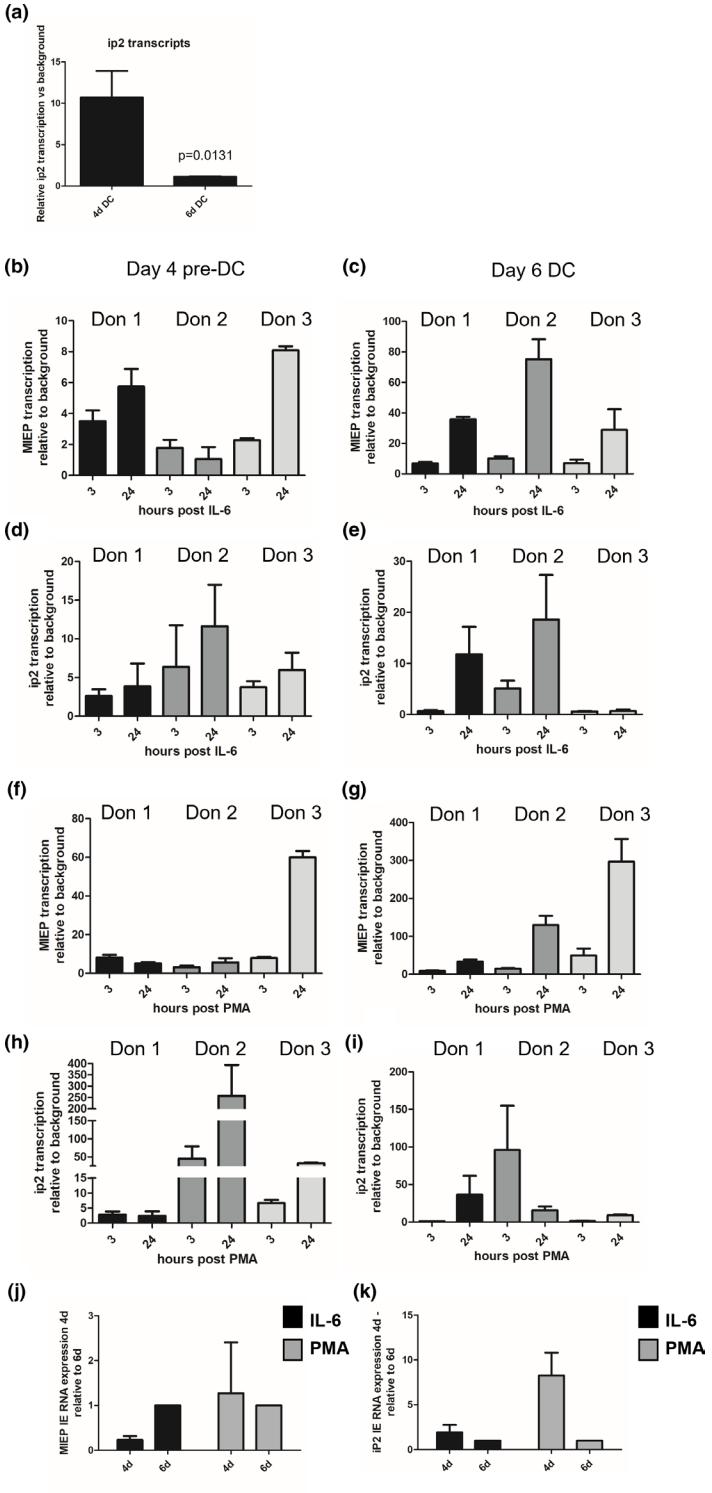

Fig. 5. The ip2 promoter is more responsive in DCs earlier in the differentiation pathway. (a) CD14+ monocytes were infected with HCMV and then at 3 days post-infection (dpi) differentiated towards a DC phenotype with IL-4/GM-CSF. RNA was collected at 4 and 6 days into differentiation and analysed for ip2 transcription which was then expressed relative to baseline background generated from a negative control signal. (b–i) CD14+ monocytes from three donors (Don 1, Don 2 and Don 3) were infected and then at 3 dpi differentiated towards a DC phenotype with IL-4/GM-CSF. RNA was collected at 4 and 6 days into differentiation and analysed for ip2 and MIEP transcription at 3 and 24 hpi stimulation with IL-6 (b–e) or PMA (f–i). For all analyses RNA expression is measured compared to a baseline background signal from a negative control signal. Each donor was analysed in two independent experiments. Error bars represent ±1 SD from the mean. (j,k) The cumulative data from each donor at both time points for MIEP (j) or ip2 (k) transcripts were analysed with the 4 day data expressed relative to 6 days (*n*=6). Error bars represent ±1 SD from the mean.

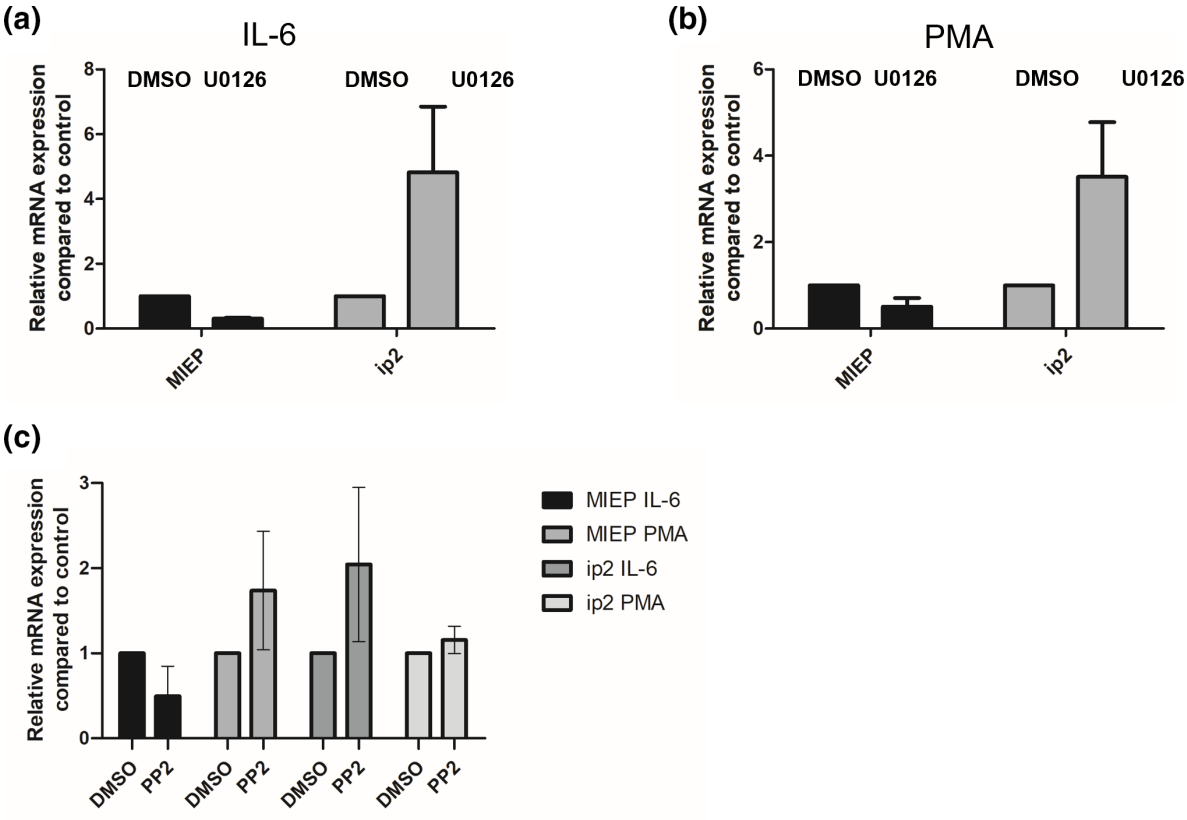

Fig. 6. ERK and SFK inhibitors repress MIEP but not ip2 activity. CD14+ monocytes were infected with HCMV and then at 3 dpi differentiated towards a DC phenotype with IL-4/GM-CSF. At 4 days post-differentiation cells were pre-treated with DMSO (a-c), ERK inhibitor U0126 (a,b) or SFK inhibitor PP2 (c) prior to stimulation with IL-6 or PMA and analysed for MIEP and ip2 transcription with U0126 or PP2 being expressed relative to the DMSO control. Unless otherwise stated, *n*=3. Error bars represent ±1 SD from the mean.

DISCUSSION

Herpes virus latency and reactivation is inextricably linked to chromatin-mediated regulation of gene expression. This relationship underpins strategies to purge latent infections from hosts using so-called 'shock and kill' therapies that rely on pharmacological manipulation of epigenetic modifications in viral DNA [48]. Furthermore, efficient gene expression during lytic infection is also sensitive to the effect of epigenetic modifiers but these small effects are clearer at lower m.o.i. Here we report on a study that identified that putative HAT inhibitors had a only minor impact on lytic infection when compared to previous studies of HATi in reactivation. Whilst we remain cautious of our interpretation of the data and, specifically, the precise contribution of HATs during lytic infection, the data do suggest that not one specific HATi had a major effect at a higher m.o.i. This could possibly reflect redundancy between the HAT families or that, potentially, the key role of HATs is in reversing MIEP repression at lower m.o.i where a tip60 phenotype was observed. However, in characterizing the impact of HATi on lytic infection, we serendipitously identified a potentially complex relationship between the MIEP and ip2 promoter activity with implications for understanding HCMV latency and reactivation.

An intriguing aspect of the work is the differential sensitivity of the MIEP and ip2 promoters to HAT inhibitors, which potentially explains the complex phenotypes we previously observed in studies interrogating the activity of epigenetic modifiers in latency and HCMV reactivation. For example, we previously reported that addition of HDACi to latently infected cells – which has been shown by many groups to activate IE gene expression – appeared to disproportionately impact MIEP activity versus ip2 activity in myeloid cells latently infected with HCMV [24], which could be explained by the recent link reported between the regulation of the MIEP, CREB response elements and HDACi [26]. Furthermore, upon reactivation, MIEP transcriptional activity was again identified to be more sensitive to the action of HATi compared to the alternative ip2 promoter, at least in the THP1 cell line model of latency [24]. The reasons for this are unclear but it might suggest that chromatin within the MIE coding region (where ip2 resides) is euchromatin-like even during latency. Alternatively, it is possible that the MIE region downstream of the MIEP (around ip2) is relatively nucleosome-free – indeed a study of the HCMV genome during lytic infection has suggested that the MIE coding region has limited evidence of nucleosome deposition [49] consistent with an earlier study that suggested

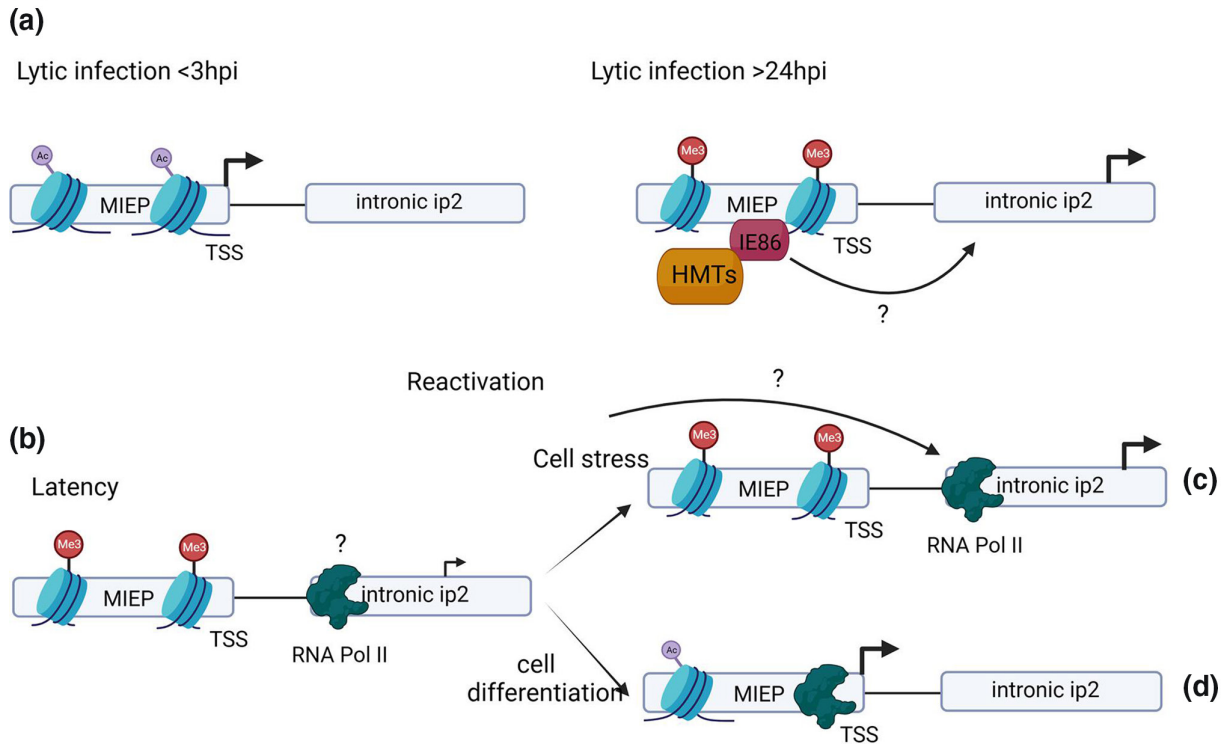

Fig. 7. Model for interdependent activity of the MIE promoters during different phases of infection. (a) Following infection of permissive cells histones are recruited to the MIEP and by 3 hpi are heavily acetylated promoting MIEP-derived IE transcription. The resulting IE86 protein accumulation promotes auto-repression of the MIEP through binding of the crs located in the MIEP. This allows transcription from ip2 which could be additionally dependent on IE86 *trans*-activation (?) as has been reported for other early viral promoters. (b) The establishment of latency is dependent on chromatin-mediated silencing of the MIEP. Lower histone occupancy in the MIE coding region could explain RNA Pol II binding (?) reported in this region during latency. (c,d) Reactivation can be triggered by cell stress (c) or cellular differentiation (d). Cell stress (c) promotes alternative promoter (ip2) activation which is chromatin-independent and potentially via direct activation of RNA Pol II bound in the MIE region. Cellular differentiation (d) promotes chromatin remodelling and activation of the MIEP and, consequently, less transcription from ip2 similar to observations in lytic infection at IE times post-infection (a). Image created using Biorender.com.

generally low histone occupancy on the viral genome during lytic infection [32]. A lack of epigenetic repression could also explain the low-level transcriptional activity of the ip2 promoter reported in latent infection [22]. Indeed, an earlier RNA Pol II ChiP-seq analysis of latently infected cells revealed evidence of RNA Pol II binding within the MIE region of latently infected cells – although this was downstream of ip2 it may, again, suggest that the MIE region is more readily accessible to RNA Pol II occupancy [50]. In that previous study, it was hypothesized that this was responsible for the expression of a putative alternative latent gene product IE1ex4 but it could also explain low-level transcription from ip2 seen by us and others in latently infected cells (summarized in Fig. 7).

Additionally, we cannot preclude the possibility that the MIE locus downstream of the MIEP is chromatinized but not in a completely canonical manner. Work from the Kalejta laboratory has shown that the histone H3.3 variant is recruited to incoming viral genomes, suggesting that chromatinization of the HCMV genome may be different to that of host DNA [51]. Furthermore, the Trono laboratory identified that TRIM28/KAP1 activity was important for silencing of HCMV gene expression during latency, including the induction of extensive histone H3 methylation on lysine 9 at sites distal to the KAP1/TRIM28 binding sites in the viral genomes [52]. Notably, they identified hotspots for chromatinization and HP1 deposition (which did include the MIEP) which, again, would be consistent with a non-canonical distribution of nucleosomes on viral DNA. If the histones loaded onto incoming HCMV genomes represent a cell-intrinsic host defence mechanism (involving KAP1/TRIM28), it is a possibility that this may be different in structure and composition to that assembled on replicating eukaryotic DNA.

One potential inference of the transcriptional data is that the canonical MIEP and ip2 promoters could have a potentially antagonistic relationship. However, perhaps a less pervasive and fairer interpretation is that the ip2 promoter is more likely to be active when the MIEP is repressed. It is well established that the MIEP is one of the strongest promoters in cell biology and thus, when active, probably predominates over ip2 activity. Indeed, ip2 transcriptional activity is usually reported at times when the MIEP is repressed during lytic infection [21]. That said, in THP-1 and Kasumi-3 cells we and others have reported MIEP- and ip2-derived transcription in response

to PMA which could be considered a contradiction of this view [22–24, 53]. However, we cannot know whether those transcripts are occurring in the same cell (which would require single cell RNA seqencing analyses to be addressed). Our data also show ERK and SFK inhibitors appeared to have a disproportionate effect on the activity of the MIEP compared to the ip2 promoter in primary cells. This activity against the MIEP is consistent with the proposed mechanism of regulation of the MIEP by ERK and SFK is via the recruitment of chromatin-modifying enzymes [16, 18] and our previous observations that epigenetic modifiers activate the MIEP more robustly than the MIEP [24]. Further, this is also consistent with the view that, in this context, MIEP and ip2 activity are independently regulated events. Finally, we also note that ERK inhibitors enhanced ip2 transcription, which may explain previous observations that ERK inhibitors can enhance reactivation in models which have also identified ip2 promoter activity as important [25, 54, 55].

The existence of the ip2 promoter and evidence of regulatory mechanisms distinct from those controlling the MIEP raises some important evolutionary questions [25]. Deletion of the ip2 promoter had the biggest impact on HCMV when investigated in models of latency and reactivation, leading to the interpretation that HCMV reactivation was dependent on the activity of ip2 [22]. In contrast, differential regulation of the MIEP (akin to the differential regulation of eukaryotic gene expression in a cell type-specific manner) was, by extension, considered to be less important. From an evolutionary perspective, differential regulation of a key promoter (such as the MIEP) that controls lytic and latent infection seems the simplest explanation rather than the existence of a second promoter downstream required specifically for reactivation. That said, our data, along with accumulating evidence from others, do suggest that the ip2 promoter is activated independent of the MIEP in a ligand-specific way and propose that the emergence of the ip2 promoter provides an additional, and perhaps independent, mechanism for HCMV reactivation (summarized Fig. 7). An increased propensity to reactivate potentially provides more opportunity for transmission – and, thus, HCMVs with two mechanisms of reactivation (MIEP and ip2) could have a selective advantage. Indeed, the ip2 promoter has been observed to be involved in a number of model cell lines that use phorbol esters such as PMA – a broad-acting stress-inducing ligand that may suggest importance in a context-specific way. Furthermore, FOXO transcription factors have been implicated as triggers of ip2 promoter activity [23] and their induction is often observed in response to stress insults as a mechanism to ameliorate events that promote cell toxicity [56, 57]. Indeed, Krishna and colleagues reported an AP-1 dependence for ip2 activity in a Kasumi-3 model of HCMV [53] which also utilizes phorbol ester-induced reactivation. Interestingly, AP-1 is activated by canonical mitogen-activated pathways but is also activated rapidly by multiple stress pathways in an Elk-1-dependent but MAPK-independent manner [58].

In conclusion, we have observed that HATi have limited impact on early stages of lytic infection with more activity at low m.o.i. and it is probable that post-IE-mediated effects are responsible for the defects in viral replication observed with these HAT inhibitors. Intriguingly, investigation of the MIE transcriptional phenotype in lytic infection suggested a potentially complex relationship of inter-dependent activity exists between the MIEP and ip2 promoters which and may help to reconcile studies from different groups regarding the importance of the MIEP versus ip2 in different models of HCMV reactivation. We propose that this increased complexity within the MIE region provides HCMV with multiple strategies to reactivate depending on the context and the stimulus for the reactivation and this could have an evolutionary advantage *in vivo*. Indeed, this responsiveness to multiple reactivation cues could underpin the propensity of this virus to emerge quickly in clinical settings, leading it to be a major cause of morbidity in multiple patient populations [1].

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Conflicts of interest
All authors declare no conflict of interest.

Ethical statement
CD14+ cells were obtained from venous blood taken from healthy volunteers who gave informed consent (NHS London Hampstead research ethic committee: 08/H0720/46). All studies with human material abide by Declaration of Helsinki principles.

References

1. Griffiths P, Reeves M. Pathogenesis of human cytomegalovirus in the immunocompromised host. *Nat Rev Microbiol* 2021;19:759–773.
2. Dupont L, Reeves MB. Cytomegalovirus latency and reactivation: recent insights into an age old problem. *Rev Med Virol* 2016;26:75–89.
3. Limaye AP, Boeckh M. CMV in critically ill patients: pathogen or bystander? *Rev Med Virol* 2010;20:372–379.
4. Legendre C, Pascual M. Improving outcomes for solid-organ transplant recipients at risk from cytomegalovirus infection: late-onset disease and indirect consequences. *Clin Infect Dis* 2008;46:732–740.
5. Meier JL, Stinski MF. Regulation of human cytomegalovirus immediate-early gene expression. *Intervirology* 1996;39:331–342.
6. Sinclair J. Chromatin structure regulates human cytomegalovirus gene expression during latency, reactivation and lytic infection. *Biochim Biophys Acta* 2010;1799:286–295.
7. Reeves MB, MacAry PA, Lehner PJ, Sissons JGP, Sinclair JH. Latency, chromatin remodeling, and reactivation of human cytomegalovirus in the dendritic cells of healthy carriers. *Proc Natl Acad Sci U S A* 2005;102:4140–4145.
8. Reeves MB, Lehner PJ, Sissons JGP, Sinclair JH. An in vitro model for the regulation of human cytomegalovirus latency and reactivation in dendritic cells by chromatin remodelling. *J Gen Virol* 2005;86:2949–2954.

9. Krishna BA, Miller WE, O'Connor CM. US28: HCMV's Swiss Army Knife. *Viruses* 2018;10:445.
10. Krishna BA, Poole EL, Jackson SE, Smit MJ, Wills MR, *et al*. Latency-associated expression of human cytomegalovirus US28 attenuates cell signaling pathways to maintain latent infection. *mBio* 2017;8:e01754-17.
11. Hahn G, Jores R, Mocarski ES. Cytomegalovirus remains latent in a common precursor of dendritic and myeloid cells. *Proc Natl Acad Sci*1998;95:3937–3942.
12. Taylor-Wiedeman J, Sissons P, Sinclair J. Induction of endogenous human cytomegalovirus gene expression after differentiation of monocytes from healthy carriers. *J Virol* 1994;68:1597–1604.
13. Reeves MB, Compton T. Inhibition of inflammatory interleukin-6 activity via extracellular signal-regulated kinase-mitogen-activated protein kinase signaling antagonizes human cytomegalovirus reactivation from dendritic cells. *J Virol* 2011;85:12750–12758.
14. Hargett D, Shenk TE. Experimental human cytomegalovirus latency in CD14+ monocytes. *Proc Natl Acad Sci U S A* 2010;107:20039–20044.
15. Prösch S, Wuttke R, Krüger DH, Volk H-D. NF-kappaB--a potential therapeutic target for inhibition of human cytomegalovirus (re) activation? *Biol Chem* 2002;383:1601–1609.
16. Kew VG, Yuan J, Meier J, Reeves MB. Mitogen and stress activated kinases act co-operatively with CREB during the induction of human cytomegalovirus immediate-early gene expression from latency. *PLoS Pathog* 2014;10:e1004195.
17. Reeves MB. Cell signaling and cytomegalovirus reactivation: what do Src family kinases have to do with it? *Biochem Soc Trans* 2020;48:667–675.
18. Dupont L, Du L, Poulter M, Choi S, McIntosh M, *et al*. Src family kinase activity drives cytomegalovirus reactivation by recruiting MOZ histone acetyltransferase activity to the viral promoter. *J Biol Chem* 2019;294:12901–12910.
19. Krishna BA, Lau B, Jackson SE, Wills MR, Sinclair JH, *et al*. Transient activation of human cytomegalovirus lytic gene expression during latency allows cytotoxic T cell killing of latently infected cells. *Sci Rep* 2016;6:24674.
20. Groves IJ, Jackson SE, Poole EL, Nachshon A, Rozman B, *et al*. Bromodomain proteins regulate human cytomegalovirus latency and reactivation allowing epigenetic therapeutic intervention. *Proc Natl Acad Sci U S A* 2021;118:e2023025118.
21. Arend KC, Ziehr B, Vincent HA, Moorman NJ. Multiple transcripts encode full-length human Cytomegalovirus IE1 and IE2 proteins during lytic infection. *J Virol* 2016;90:8855–8865.
22. Collins-McMillen D, Rak M, Buehler JC, Igarashi-Hayes S, Kamil JP, *et al*. Alternative promoters drive human cytomegalovirus reactivation from latency. *Proc Natl Acad Sci U S A* 2019;116:17492–17497.
23. Hale AE, Collins-McMillen D, Lenarcic EM, Igarashi S, Kamil JP, *et al*. FOXO transcription factors activate alternative major immediate early promoters to induce human cytomegalovirus reactivation. *Proc Natl Acad Sci U S A* 2020;117:18764–18770.
24. Mason R, Groves IJ, Wills MR, Sinclair JH, Reeves MB. Human cytomegalovirus major immediate early transcripts arise predominantly from the canonical major immediate early promoter in reactivating progenitor-derived dendritic cells. *J Gen Virol* 2020;101:635–644.
25. Collins-McMillen D, Kamil J, Moorman N, Goodrum F. Control of immediate early gene expression for human Cytomegalovirus reactivation. *Front Cell Infect Microbiol* 2020;10:476.
26. Albright ER, Walter RM, Saffert RT, Kalejta RF. NFκB and cyclic AMP response element sites mediate the valproic acid and UL138 responsiveness of the human Cytomegalovirus major immediate early enhancer and promoter. *J Virol* 2023;97:e0002923.
27. Keller MJ, Wu AW, Andrews JI, McGonagill PW, Tibesar EE, *et al*. Reversal of human cytomegalovirus major immediate-early enhancer/promoter silencing in quiescently infected cells via the cyclic AMP signaling pathway. *J Virol* 2007;81:6669–6681.
28. Keller MJ, Wheeler DG, Cooper E, Meier JL. Role of the human cytomegalovirus major immediate-early promoter's 19-base-pair-repeat cyclic AMP-response element in acutely infected cells. *J Virol* 2003;77:6666–6675.
29. Krishna BA, Wass AB, Dooley AL, O'Connor CM. CMV-encoded GPCR pUL33 activates CREB and facilitates its recruitment to the MIE locus for efficient viral reactivation. *J Cell Sci* 2021;134:jcs254268.
30. Reeves MB. Chromatin-mediated regulation of cytomegalovirus gene expression. *Virus Res* 2011;157:134–143.
31. Cuevas-Bennett C, Shenk T. Dynamic histone H3 acetylation and methylation at human cytomegalovirus promoters during replication in fibroblasts. *J Virol* 2008;82:9525–9536.
32. Nitzsche A, Paulus C, Nevels M. Temporal dynamics of cytomegalovirus chromatin assembly in productively infected human cells. *J Virol* 2008;82:11167–11180.
33. Groves IJ, Reeves MB, Sinclair JH. Lytic infection of permissive cells with human cytomegalovirus is regulated by an intrinsic "pre-immediate-early" repression of viral gene expression mediated by histone post-translational modification. *J Gen Virol* 2009;90:2364–2374.
34. Michaelis M, Suhan T, Reinisch A, Reisenauer A, Fleckenstein C, *et al*. Increased replication of human cytomegalovirus in retinal pigment epithelial cells by valproic acid depends on histone deacetylase inhibition. *Invest Ophthalmol Vis Sci* 2005;46:3451–3457.
35. Woodhall DL, Groves IJ, Reeves MB, Wilkinson G, Sinclair JH. Human Daxx-mediated repression of human cytomegalovirus gene expression correlates with a repressive chromatin structure around the major immediate early promoter. *J Biol Chem* 2006;281:37652–37660.
36. Nevels M, Paulus C, Shenk T. Human cytomegalovirus immediate-early 1 protein facilitates viral replication by antagonizing histone deacetylation. *Proc Natl Acad Sci U S A* 2004;101:17234–17239.
37. Mocarski ES, Kemble GW, Lyle JM, Greaves RF. A deletion mutant in the human cytomegalovirus gene encoding IE1(491aa) is replication defective due to A failure in autoregulation. *Proc Natl Acad Sci U S A* 1996;93:11321–11326.
38. Vogel JL, Kristie TM. The dynamics of HCF-1 modulation of herpes simplex virus chromatin during initiation of infection. *Viruses* 2013;5:1272–1291.
39. Gan X, Wang H, Yu Y, Yi W, Zhu S, *et al*. Epigenetically repressing human cytomegalovirus lytic infection and reactivation from latency in THP-1 model by targeting H3K9 and H3K27 histone demethylases. *PLoS One* 2017;12:e0175390.
40. Liang Y, Vogel JL, Narayanan A, Peng H, Kristie TM. Inhibition of the histone demethylase LSD1 blocks alpha-herpesvirus lytic replication and reactivation from latency. *Nat Med* 2009;15:1312–1317.
41. Reeves M, Murphy J, Greaves R, Fairley J, Brehm A, *et al*. Autorepression of the human cytomegalovirus major immediate-early promoter/enhancer at late times of infection is mediated by the recruitment of chromatin remodeling enzymes by IE86. *J Virol* 2006;80:9998–10009.
42. Cherrington JM, Khoury EL, Mocarski ES. Human cytomegalovirus ie2 negatively regulates alpha gene expression via a short target sequence near the transcription start site. *J Virol* 1991;65:887–896.
43. Lee KK, Workman JL. Histone acetyltransferase complexes: one size doesn't fit all. *Nat Rev Mol Cell Biol* 2007;8:284–295.
44. Elmallah MIY, Micheau O. Epigenetic Regulation of TRAIL Signaling: Implication for Cancer Therapy. *Cancers* 2019;11:850.
45. Li R, Zhu J, Xie Z, Liao G, Liu J, *et al*. Conserved herpesvirus kinases target the DNA damage response pathway and TIP60 histone acetyltransferase to promote virus replication. *Cell Host Microbe* 2011;10:390–400.
46. Bryant LA, Mixon P, Davidson M, Bannister AJ, Kouzarides T, *et al*. The human cytomegalovirus 86-kilodalton major immediate-early protein interacts physically and functionally with histone acetyltransferase P/CAF. *J Virol* 2000;74:7230–7237.

47. Chimenti F, Bizzarri B, Maccioni E, Secci D, Bolasco A, *et al*. A novel histone acetyltransferase inhibitor modulating Gcn5 network: cyclopentylidene-[4-(4'-chlorophenyl)thiazol-2-yl)hydrazone. *J Med Chem* 2009;52:530–536.
48. Perera MR, Wills MR, Sinclair JH. HCMV antivirals and strategies to target the latent reservoir. *Viruses* 2021;13:817.
49. Spector BM, Parida M, Li M, Ball CB, Meier JL, *et al*. Differences in RNA polymerase II complexes and their interactions with surrounding chromatin on human and cytomegalovirus genomes. *Nat Commun* 2022;13:2006.
50. Tarrant-Elorza M, Rossetto CC, Pari GS. Maintenance and replication of the human cytomegalovirus genome during latency. *Cell Host Microbe* 2014;16:43–54.
51. Albright ER, Kalejta RF. Canonical and variant forms of histone H3 are deposited onto the human cytomegalovirus genome during lytic and latent infections. *J Virol* 2016;90:10309–10320.
52. Rauwel B, Jang SM, Cassano M, Kapopoulou A, Barde I, *et al*. Release of human cytomegalovirus from latency by a KAP1/TRIM28 phosphorylation switch. *eLife* 2015;4:e06068.
53. Krishna BA, Wass AB, O'Connor CM. Activator protein-1 transactivation of the major immediate early locus is a determinant of cytomegalovirus reactivation from latency. *Proc Natl Acad Sci U S A* 2020;117:20860–20867.
54. Buehler J, Carpenter E, Zeltzer S, Igarashi S, Rak M, *et al*. Host signaling and EGR1 transcriptional control of human cytomegalovirus replication and latency. *PLoS Pathog* 2019;15:e1008037.
55. Buehler J, Zeltzer S, Reitsma J, Petrucelli A, Umashankar M, *et al*. Opposing regulation of the EGF receptor: a molecular switch controlling cytomegalovirus latency and replication. *PLoS Pathog* 2016;12:e1005655.
56. Akasaki Y, Alvarez-Garcia O, Saito M, Caramés B, Iwamoto Y, *et al*. FoxO transcription factors support oxidative stress resistance in human chondrocytes. *Arthritis Rheumatol* 2014;66:3349–3358.
57. Sengupta A, Molkentin JD, Paik J-H, DePinho RA, Yutzey KE. FoxO transcription factors promote cardiomyocyte survival upon induction of oxidative stress. *J Biol Chem* 2011;286:7468–7478.
58. Gille H, Strahl T, Shaw PE. Activation of ternary complex factor Elk-1 by stress-activated protein kinases. *Curr Biol* 1995;5:1191–1200.