Activator protein-1 transactivation of the major immediate early locus is a determinant of cytomegalovirus reactivation from latency

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Author Contributions

B.A.K and C.M.O. designed the research; B.A.K. and A.B.W. performed the research; B.A.K, A.B.W., and C.M.O. contributed new reagents/analytic tools; B.A.K., A.B.W., and C.M.O. analyzed data; B.A.K and C.M.O. wrote the paper.

Abstract

Human cytomegalovirus (HCMV) is a ubiquitous pathogen that latently infects hematopoietic cells and has the ability to reactivate when triggered by immunological stress. This reactivation causes significant morbidity and mortality in immune-deficient patients, who are unable to control viral dissemination. While a competent immune system helps retain HCMV in its latent form, a portrait of the factors that induce reactivation following the proper cues remains incomplete. Our understanding of the complex molecular mechanisms underlying latency and reactivation continue to evolve. We previously showed the HCMV-encoded G-protein coupled receptor *US28* is expressed during latency and facilitates latent infection by attenuating the activator protein-1 (AP-1) transcription factor subunit, c-fos, expression and activity. We now show AP-1 is a critical component for HCMV reactivation. Pharmacological inhibition of c-fos significantly attenuates viral reactivation. In agreement, infection with a virus in which we disrupted the proximal AP-1 binding site in the major immediate early (MIE) enhancer results in inefficient reactivation compared to wild type. Concomitantly, AP-1 recruitment to the MIE enhancer is significantly decreased following reactivation of the mutant virus. Further, AP-1 is critical for de-repression of MIE-driven transcripts and downstream early and late genes, while immediate early genes from other loci remain unaffected. Our data also reveal MIE transcripts driven from the MIE promoter, the distal promoter, and the internal promoter, iP2, are dependent upon AP-1 recruitment, while iP1-driven transcripts are AP-1-independent. Collectively, our data demonstrate AP-1 binding to and activation of the MIE enhancer is a key molecular process controlling reactivation from latency.

Significance Statement

Human cytomegalovirus (HCMV) is a common pathogen that infects the majority of the population for life. This infection poses little threat in immunologically healthy individuals, but can be fatal in people with compromised immune systems. Our understanding of the mechanisms underlying latency and reactivation remains incomplete. Here, we show the cellular transcription factor, AP-1, is a key to regulating HCMV reactivation. Our findings reveal AP-1 binding to the major immediate early enhancer/promoter is critical for switching this locus from one that is repressed during latency to one that is highly active following reactivation. Our work

provides a novel mechanism HCMV exploits to reactivate, highlighting AP-1 as a potential target to prevent HCMV reactivation.

Main Text

Introduction

The betaherpesvirus human cytomegalovirus (HCMV) latently infects 50 - 75% of the healthy US population (1). While this infection is, for the most part, asymptomatic in healthy individuals, reactivation from latency causes significant morbidity and mortality in immunocompromised and immunosuppressed individuals (2, 3). Reactivation is triggered by immunodeficiency combined with differentiation of latently infected myeloid cells (4, 5) and is treated with antiviral therapies, such as valganciclovir and foscarnet (6). These antiviral compounds are limited by toxicity and the emergence of drug resistant viral strains. Further, these drugs target viral lytic replication, when disease is already primed to occur (7, 8). Thus, novel approaches aimed at preventing viral reactivation will prove beneficial for immunodeficient patients at risk for HCMV-associated diseases.

Latent infection is defined as the maintenance of viral genomes in the absence of infectious virus production, coupled with the potential to reactivate given the proper extracellular and/or environmental cues (9). A critical determinant in the establishment and maintenance of HCMV latency is repression of the major immediate early promoter (MIEP). The MIEP is a powerful promoter, which drives the expression of the major immediate early (MIE) genes, *UL122* and *UL123* that encode IE2 and IE1, respectively. These MIE proteins transactivate other sites of E gene expression, which in turn facilitate the lytic life cycle. As such, the MIE locus is significantly repressed during viral latency (10-12). This silencing is mediated, at least in part, by the recruitment of both repressive transcription factors and chromatin modifiers to the MIE enhancer/promoter region (13). During reactivation from latency, these repressive factors are overcome, resulting in de-repression of the MIE enhancer/promoter and expression of the MIE-driven genes. This likely requires a multifaceted process that exchanges pro-latency factors for transcription factors and chromatin remodeling favoring the re-expression of IE1 and IE2 (13, 14). It is also clear that additional promoters within the MIE region drive transcription of mRNAs with differing 5' untranslated region (UTR) lengths, all of which can encode full length IE1 and

82 IE2 proteins. Two of these non-canonical transcripts are derived from promoters within intron A
83 of the MIE region, termed iP1 and iP2 (15). Interestingly, recent work from Collins-McMillen, et
84 al. demonstrated iP2-derived transcripts are the most abundant during both latency and
85 reactivation. Furthermore, transactivation of both iP1 and iP2 are required for efficient viral
86 reactivation in primary $CD34^+$ hematopoietic progenitor cells (HPCs) (16), though the regulation
87 of these newly identified promoters remains unknown. These findings highlight the complexity of
88 the transcriptional control of the MIE locus.

89 Work from many investigators implicates multiple factors in de-repressing the MIE locus
90 during reactivation. These include: src family kinase activation of extracellular signal-regulated
91 kinase/mitogen-activated protein kinase (ERK/MAPK; ref. (17), cyclic AMP (cAMP) activation
92 (18), cAMP response element-binding protein (CREB) phosphorylation (19), epidermal growth
93 factor receptor (EGFR) activation of phosphoinositide 3-kinase (PI3K; ref. (20), facilitates
94 chromatin transcription (FACT)-mediated transactivation (21), mammalian target of rapamycin
95 (mTOR)-modulated activation of KRAB-associated protein-1 (KAP1; ref. (22), and protein kinase
96 A (PKA)-CREB-TORC2 transactivation (23). Although these results summarize findings from a
97 variety of latency model systems, they underscore the idea that reactivation likely requires
98 concomitant signals from multiple pathways and factors to de-repress the MIE locus, triggering
99 epigenetic changes as well as the recruitment of transcription factors that transactivate this
100 region.

101 We recently showed *US28*, an HCMV encoded G-protein coupled receptor (GPCR),
102 attenuates cellular fos (c-fos) signaling to maintain latency (24, 25). Dimerization of c-fos and c-
103 jun form the transcription factor, activator protein-1 (AP-1), which binds and transactivates the
104 MIEP in luciferase reporter assays (26). US28 protein (pUS28) suppression of phosphorylated
105 c-fos prevents AP-1 from binding the MIE enhancer and activating the MIE locus during latent
106 infection of Kasumi-3 cells (a $CD34^+$ cell line that supports latency and viral reactivation) and
107 primary *ex vivo* $CD34^+$ hematopoietic progenitor cells (HPCs) (25). The MIE enhancer region
108 contains two AP-1 binding sites: the promoter proximal binding site has a canonical AP-1
109 binding motif, while the promoter distal binding site has a non-consensus sequence (26, 27).
110 Disruption of both binding sites together does not impact lytic replication in fibroblasts or
111 epithelial cells, nor does it affect virulence in a chimeric murine CMV infection model (26, 27),
112 suggesting AP-1 recruitment to the MIEP is dispensable for lytic infection.

113 Our previous data revealed suppressing AP-1 activity is necessary for maintaining
114 HCMV latency (25), and thus we hypothesized AP-1 recruitment to the MIEP is important for

115 efficient reactivation from latency in hematopoietic cells. Herein, we show pharmacological
116 inhibition of AP-1 in the presence of reactivation stimuli decreases reactivation efficiency.
117 Additionally, mutation of the promoter proximal AP-1 binding site attenuates AP-1 recruitment to
118 the MIE enhancer and impairs reactivation following stimuli. Finally, our data reveal AP-1
119 recruitment to the MIE enhancer during reactivation is required to stimulate the expression of
120 MIE-driven genes originating from the canonical MIEP, iP2, and the distal promoter (dP), but not
121 other IE genes or iP1-driven transcripts. Taken together, our results show AP-1 transactivation
122 of the MIE enhancer/promoter is an important factor for efficient HCMV reactivation from
123 latency.
124

125 **Results**

126 **Pharmacological inhibition of c-fos suppresses viral reactivation in Kasumi-3 cells**

127 We previously showed HCMV suppresses AP-1 activation to maintain latency (25), which led us
128 to posit the requirement for this transcription factor's activation during reactivation from latency.
129 To test the impact AP-1 signaling has on reactivation, we infected Kasumi-3 cells with
130 TB40/E*mCherry* (WT) and cultured the cells in conditions favoring latency for 7 days (d).
131 Cultures were then treated with vehicle (dimethylsulfoxide, DMSO) to maintain latency or
132 tetradecanoyl phorbol acetate (TPA) to induce reactivation for an additional 2 d in the presence
133 or absence of the c-fos inhibitor, T-5224, at a concentration we previously showed does not
134 impact cell viability (25). We assessed the ability of each infection to reactivate by co-culturing
135 the infected Kasumi-3 cells with naïve fibroblasts and quantified infectious virus production by
136 Extreme Limiting Dilution Analysis (ELDA). As expected, TPA treatment of infected Kasumi-3
137 cells induced reactivation, as measured by an increase in virus production (28). However,
138 treatment with T-5224 attenuated virus production in TPA-treated cells (Fig. 1A). We next
139 confirmed this finding using cord blood-derived, *ex vivo* cultured $CD34^+$ HPCs, a natural site of
140 HCMV latency. We infected these primary cells with WT virus for 7 d under conditions favoring
141 latency, after which we cultured a portion of the infected cells in media that promotes
142 reactivation (29) either with or without T-5224. Similar to Kasumi-3 cells, our findings reveal
143 treatment with T-5224 reduced virus production in $CD34^+$ HPCs treated with reactivation stimuli
144 (Fig. 1B). Together, these results indicate AP-1 activation is important for efficient reactivation
145 from latency.
146

147 **Mutation of the promoter proximal AP-1 binding site in the MIE enhancer impairs viral**
148 **reactivation in hematopoietic cells**
149 Based on our findings above, we next asked if AP-1 recruitment to the MIE enhancer aids in de-
150 repressing the MIE locus during HCMV reactivation. To begin to investigate the contribution of
151 AP-1 to this process, we generated a recombinant virus in the bacterial artificial chromosome
152 (BAC)-derived, clinical isolate TB40/E (30), which we previously engineered to express mCherry
153 (TB40/E*mCherry*) (31). We disrupted the promoter proximal AP-1 binding site to generate
154 TB40/E*mCherry*-proximal-AP-1*mutant* (AP-1mut_p). Additionally, to ensure we did not introduce
155 off-site mutations during the recombineering process, we restored the mutated MIE
156 enhancer/promoter region to its wild type sequence to generate the repair virus,
157 TB40/E*mCherry*-proximal-AP-1*repair* (AP-1*rep*) (SI Appendix, Fig. S1). Consistent with previous
158 findings (26, 27), we confirmed AP-1mut_p and AP-1*rep* display WT growth phenotypes in
159 fibroblasts (SI Appendix, Fig. S2A). Similarly, both the mutant and repair viruses displayed WT
160 growth following lytic infection of epithelial cells (SI Appendix, Fig. S2B). Together, these results
161 suggest AP-1 recruitment to the promoter proximal site is not required for efficient lytic
162 replication.
163 To determine the contribution of the AP-1 promoter proximal binding site during
164 reactivation from latency, we infected Kasumi-3 cells with WT, AP-1mut_p, or AP-1*rep* for 7 d
165 under conditions favoring latency, after which we performed ELDA in the presence of vehicle or
166 TPA treatment. We found AP-1mut_p-infected Kasumi-3 cells failed to efficiently reactivate to WT
167 or AP-1*rep* levels (Fig. 2A). Importantly, AP-1mut_p-infected cells maintain comparable levels of
168 the viral genome compared to WT- or AP-1*rep*-infected cultures (SI Appendix, Fig. S3),
169 suggesting these infected cultures harbor latent virus. Similarly, the latency-associated gene,
170 *UL138*, was highly expressed relative to *UL123* in all infected cultures, consistent with latent
171 infection (SI Appendix, Fig. S4). Treatment of each infected culture with TPA resulted in a
172 decrease in the ratio of *UL138*/*UL123* (SI Appendix, Fig. S4), consistent with viral reactivation
173 (24, 25, 28). The ratio of *UL138*:*UL123* neared 1 in the WT- and AP-1*rep*-infected cells following
174 TPA treatment, which was significantly lower than the ratio of these transcripts in the AP-1mut_p-
175 infected counterpart cultures (SI Appendix, Fig. S4), suggesting inefficient reactivation of the
176 mutant virus. Consistent with these observations, AP-1mut_p-infected primary $CD34^+$ HPCs
177 produces significantly less infectious virus than either WT or AP-1*rep* when stimulated to
178 reactivate (Fig. 2B). Together, these data suggest AP-1mut_p-infected hematopoietic cells
179 undergo latency, but fail to efficiently reactivate to WT levels following stimuli.

180 Finally, to distinguish between the relative contributions of AP-1 activity and the
181 requirement for the AP-1 promoter proximal binding site during reactivation from latency, we
182 evaluated viral reactivation in the presence or absence of the c-fos inhibitor, T-5224. To this
183 end, we infected Kasumi-3 cells with WT, AP-1*mut*$_p$, or AP-1*rep* for 7 d under conditions
184 favoring latency. We then quantified infectious virus production by ELDA in the presence or
185 absence of TPA and/or T-5224. In the presence of T-5224, WT- and AP-1*rep*-infected cells fail
186 to efficiently reactivate virus, resulting in virion production similar to AP-1*mut*$_p$-infected cells in
187 the absence of T-5224 treatment (Fig. 2C). Further, T-5224 did not further impair reactivation of
188 the AP-1*mut*$_p$ virus. Taken together, these results demonstrate the importance of AP-1
189 activation and the requirement for the promoter proximal site of the MIE enhancer in promoting
190 HCMV reactivation.
191
192 **Recruitment of the AP-1 transcription factor to the MIE enhancer is significantly**
193 **diminished following reactivation stimulus of AP-1*mut*$_p$-infected Kasumi-3 cells**
194 To test our hypothesis that AP-1 binds to the MIE enhancer, we first evaluated the recruitment
195 of the AP-1 subunits, c-fos and c-jun, to the enhancer region. We latently infected Kasumi-3
196 cells with WT, AP-1*mut*$_p$, or AP-1*rep* for 7 d and then treated a portion of the infected cultures
197 for an additional 2 d with TPA treatment to induce reactivation. We then harvested the cultures
198 and performed chromatin immunoprecipitation (ChIP) assays to measure binding of c-fos and c-
199 jun to the MIE enhancer. Our results show both c-fos and c-jun bind the MIE enhancer following
200 TPA treatment of WT, latently infected Kasumi-3 cells, although disruption of the promoter
201 proximal AP-1 site reduced recruitment of each AP-1 subunit (Fig. 3). While both c-fos and c-jun
202 recruitment is significantly attenuated in the AP-1*mut*$_p$-infected Kasumi-3 cells, our data reveal
203 neither AP-1 subunit is completely devoid of binding. This suggests AP-1 also binds the
204 promoter distal, non-consensus AP-1 binding site in the MIE enhancer region. Nonetheless, our
205 data collectively indicate the promoter distal AP-1 binding site plays a minor role in reactivation
206 from latency, as deletion of the promoter proximal site alone significantly impacts efficient
207 reactivation following stimuli (Fig. 2A and B) despite detectable promoter distal site binding of
208 AP-1 (Fig. 3).
209
210 **AP-1 recruitment to the MIE enhancer during reactivation initiates MIE-driven gene**
211 **expression, but not the transcription of other IE genes**

212 Together, our data reveal AP-1 recruitment to the promoter proximal site in the MIE enhancer is
213 an important determinant for efficient viral reactivation. Thus, we hypothesized this transcription
214 factor is critical for de-repression of *UL122* and *UL123* expression. In line with this, we posited
215 the recruitment of AP-1 to the MIE enhancer would not impact other IE genes. To this end, we
216 latently infected Kasumi-3 cells with WT, AP-1mut_p, or AP-1*rep* for 7 d, after which we treated a
217 portion of the cells with TPA for an additional 2 d to stimulate reactivation. We then harvested
218 total RNA from the infected cultures and analyzed HCMV gene expression by RT-qPCR. We
219 assessed the MIE-driven transcripts *UL122* and *UL123*, representative non-MIE-derived IE
220 genes (*UL36*, *UL37*), as well as a representative early (E; *UL44*) and late (L; *UL99*) transcript.
221 AP-1mut_p-infected Kasumi-3 cells treated with TPA displayed attenuated *UL122* and *UL123*
222 transcription compared to the WT- or AP-1*rep*-infected cultures (Fig. 4A and B). As expected,
223 this decreased E (*UL44*, Fig. 4E) and L (*UL99*, Fig. 4F) transcript abundance, whose
224 transactivation is dependent upon MIE-driven transcription and their subsequent translation
225 (32). However, transcription of *UL36* and *UL37*, whose expression is not regulated by the MIE
226 enhancer, was unaffected by the mutation of the promoter proximal AP-1 site (Fig. 4C and D).
227 Taken together, these data suggest AP-1 recruitment to the promoter proximal site within the
228 MIE enhancer is required for the expression of MIE-driven transcripts and efficient viral
229 reactivation.

230 Thus far, our findings indicate disruption of promoter proximal AP-1 binding to the MIE
231 enhancer impairs de-repression of MIE transcript expression. However, IE1 and IE2 are
232 translated from multiple transcripts originating from the canonical MIEP, as well as alternative
233 transcription start sites (TSSs) within the MIE region. While these alternative transcripts differ in
234 their 5'UTR, each encodes full-length IE1 or IE2 protein (15). More recently, Collins-McMillen et
235 al. found two of these alternative transcripts derived from promoters within intron A, iP1 and iP2,
236 are important for reactivation from latency (16). However, the contribution of transcription factor
237 binding on the activity of these alternative promoters during reactivation is unknown. To this
238 end, we infected Kasumi-3 cells as above and analyzed expression of MIE mRNAs by RT-
239 qPCR, using primers specific for the transcripts derived from the dP, canonical MIEP, or the
240 internal promoters, iP1 and iP2 (Fig. 5A). Following TPA-induced reactivation of WT-infected
241 cultures, transcripts originating from each promoter increased (Fig. 5B-E), concomitant with
242 *UL122* (Fig. 4A) and *UL123* (Fig. 4B) transcription. These findings are consistent with previous
243 findings using the THP-1 culture system (16). However, AP-1mut_p-infected Kasumi-3 cells
244 displayed impaired MIEP-, iP2-, and dP-derived transcripts compared to cultures infected with

245 either WT or AP-1*rep* (Fig. 5B, D, and E, respectively). In contrast to these MIE-derived
246 transcripts, mRNA driven from the iP1 promoter was expressed to similar levels following
247 reactivation when compared to WT- or AP-1*rep*-infected cultures (Fig. 5C), suggesting iP1-
248 driven transcription is independent of AP-1 binding to the promoter proximal site in the MIE
249 enhancer. Taken together, these data suggest AP-1 binding to the MIE enhancer facilitates the
250 expression of MIEP-, iP2-, and dP-derived transcripts, while iP1-driven transcription remains
251 independent of AP-1 recruitment to this region.

252

253 **Discussion**

254 HCMV reactivation is a multifaceted process, requiring de-repression of *UL122* and *UL123*
255 transcription, changes in transcription factor binding, as well as changes in chromatin structure
256 to promote transcription (17). In this study, we found AP-1 binding to the MIE enhancer is critical
257 for the re-expression of both *UL122* and *UL123*, as well as HCMV reactivation.
258 Pharmacological inhibition of c-fos, a component of AP-1, significantly reduced the reactivation
259 efficiency of WT-infected hematopoietic cells (Fig. 1). Similarly, disruption of the promoter
260 proximal AP-1 binding site within the MIE enhancer attenuated HCMV reactivation (Fig. 2). Our
261 data demonstrate AP-1 is recruited to the MIE enhancer following reactivation of WT virus, and
262 mutation of the promoter proximal AP-1 binding significantly decreases AP-1 binding (Fig. 3).
263 Further, while the MIE enhancer has two AP-1 binding sites, our data emphasize the promoter
264 proximal site is critical for viral reactivation (Fig. 2, 3). AP-1 recruitment to the MIE enhancer
265 aids in the transactivation of the MIE-driven IE genes, *UL122* and *UL123*, while IE genes not
266 derived from the MIE region, including *UL36* and *UL37*, remain unaffected (Fig. 4). Importantly,
267 AP-1 binding to its promoter proximal site in the MIE enhancer activates transcription from the
268 canonical MIEP, as well as the alternative iP2 and dP promoters, while iP1 activity is AP-1-
269 independent (Fig. 5). Together, our results reveal a mechanism by which the AP-1 transcription
270 factor binds the MIE enhancer to activate multiple promoters in the MIE locus, thereby
271 contributing to successful reactivation of HCMV from latency.

272 While viral reactivation is likely multifactorial, it is clear c-fos and c-jun are critical
273 components of this process. Our previous findings showed pUS28 attenuates c-fos expression
274 and activation, concomitant with reduced AP-1 recruitment to the MIE enhancer during latency
275 (25). Additionally, we have shown c-jun expression is significantly suppressed during HCMV

276 latency (33), although this is not contingent upon pUS28 expression or function (25). These
277 findings suggest HCMV has adapted independent mechanisms to attenuate the two subunits
278 comprising AP-1 to prevent its binding to the MIE enhancer during latency.

279 Our findings also suggest HCMV evolved sites for AP-1 recruitment to the MIE enhancer
280 region for viral reactivation, although this association is not required for efficient lytic replication.
281 This is not unprecedented, as similar mechanisms were described for the CRE response
282 elements (19, 34). Consistent with this, several groups have shown AP-1 binds to and activates
283 the MIE enhancer/promoter in *in vitro* expression assays (26, 34, 35). In agreement with our
284 results herein, AP-1 is recruited to the MIE enhancer in a murine model of allograft-induced
285 murine CMV (MCMV) reactivation (36). Furthermore, work from Isern, et al, revealed disruption
286 of both AP-1 binding sites within the MIE enhancer region had no effect on HCMV lytic
287 replication in fibroblast and epithelial cells (26), consistent with our current findings. There is,
288 however a notable difference between this study and ours. Isern, et al. generated a chimeric
289 MCMV, replacing the native murine MIE enhancer with the HCMV enhancer region, and then
290 disrupted both AP-1 binding sites. Using this chimeric recombinant in either lung or spleen
291 explants of neonatal mice, they found no difference in virus reactivation relative to infection with
292 a chimeric virus containing wild type AP-1 binding sites (26). While this work revealed no role for
293 the promoter proximal AP-1 binding site, there are significant differences in the experimental
294 models we employed herein and the chimeric MCMV-infected mouse models used in the
295 previously published study (26).

296 In addition to HCMV, other herpesviruses modulate AP-1 during infection. The Epstein
297 Barr virus (EBV) protein, BGLF2, and the Kaposi's sarcoma-associated herpesvirus (KSHV)-
298 encoded ORF45 both activate the AP-1 signaling pathway to promote viral gene expression,
299 replication, and survival (37, 38). EBV also encodes an AP-1 homolog, BZLF1, which, like AP-1,
300 supports resting B cell proliferation and binds methylated EBV promoters critical for reactivation
301 (39). Murine gammaherpesvirus 68 (MHV68) expresses the microRNA mghv-miR-M1-1, which
302 downregulates c-jun and subsequent AP-1 activation, resulting in the suppression of viral lytic
303 replication (40). Together, these results show controlling AP-1 is a common strategy among
304 herpesviruses, suggesting targeting this transcription factor could prove beneficial for
305 suppressing reactivation and subsequent disease associated with a variety of herpesviruses.

306 Recently published findings from Collins-McMillen, et al. detailed the requirement for
307 iP1- and iP2-derived transcripts for viral reactivation (16). Our findings build upon these
308 observations and begin to unravel the likely complex web of transcription factors that regulate

309 the iP1 and iP2 promoters. We found AP-1 recruitment to the promoter proximal site within the
310 MIE enhancer drives transcription from the MIEP, iP2, and dP (Fig. 5B, D, and E, respectively).
311 However, transcription from iP1 is independent of AP-1 recruitment to the promoter proximal
312 site within the MIE enhancer, as the AP-1*mut*$_p$-infected cells express iP1-driven transcripts to
313 levels similar to those we observed in WT-infected cells (Fig. 5C). This finding suggests iP1-
314 driven transcription alone is not sufficient to drive reactivation, as AP-1*mut*$_p$-infected cells fail to
315 efficiently produce infectious virus following the addition of reactivation stimuli (Fig. 2). This
316 result also suggests additional transcription factors or chromatin remodeling proteins may
317 alternatively control iP1 activity, further highlighting the complexity in the regulation of
318 transcription in MIE locus.

319 How is AP-1 activated in response to cues that reactivate HCMV? Elucidating the
320 precise biological mechanism could indeed reveal potential therapeutic targets one could exploit
321 to prevent viral reactivation. Two pathways that activate AP-1, Src-ERK (41) and PI3K/Akt (42),
322 are already implicated in HCMV reactivation (17, 20) and are therefore potential candidates for
323 further investigation. As a potent signaling molecule, US28 is an attractive, latently expressed
324 viral protein capable of modulating cellular signaling pathways such as these. While our
325 previous data showed US28 attenuates c-fos expression and activity as well as AP-1 binding to
326 the MIE enhancer during latency (25), it remains unknown how US28 signaling is modified to
327 favor reactivation rather than latent conditions. It is attractive to hypothesize an additional viral
328 or cellular protein is triggered to alter US28's behavior following reactivation stimuli, though
329 additional work is needed to realize such mechanistic nuances. It is also possible AP-1 recruits
330 other factors, such as histone acetyltransferases (HATs) (43) to facilitate chromatin remodeling
331 and/or other transcription factors (e.g. CREB and NFκB), all of which would contribute to de-
332 repression of MIE promoters (13). Finally, c-fos and c-jun functional activity contribute to
333 myeloid differentiation and function in response to pro-inflammatory stimuli (44). These
334 possibilities present numerous avenues for future research aimed at understanding the
335 underlying biological mechanisms regulating AP-1 activation and its transactivation of the MIE
336 enhancer/promoter region.

337 Our findings detailed herein reveal AP-1 activation is critical for HCMV reactivation. AP-1
338 recruitment to its promoter proximal site within the MIE enhancer activates multiple MIE
339 promoters, including the dP, MIEP, and iP2, to drive MIE transcription, which overall facilitates
340 reactivation. Herein, we provide a novel mechanism underlying MIE de-repression during HCMV
341 reactivation, further illuminating the molecular details essential for understanding HCMV latency

342 and reactivation that will lead to the development of novel therapies to prevent viral reactivation
343 in vulnerable patients.
344
345 **Methods**

346 **Cells and Viruses**. Details regarding the cells and their culture conditions, as well as details on
347 the propagation of the TB40/E BAC-derived viruses are included in the SI Appendix, SI
348 Methods.

349 **DNA, RNA, and Protein Analyses**. Experimental approaches are included in the SI Appendix,
350 SI Methods.

351 **Infection of Kasumi-3 and CD34$^+$ Cells**. Kasumi-3 cells were infected as described previously
352 (28). Briefly, cells were serum-starved in X-VIVO15 (Lonza) 48 hours (h) before infection and
353 then infected at a multiplicity of 1.0 50% tissue culture infectious dose ($TCID_{50}$)/cell by
354 centrifugal enhancement (1,000 × *g*, 35 minutes [min], room temperature) at 5 x 10^5 cells/ml in
355 X-VIVO15. The following day, cells were treated with trypsin to remove any virus that had not
356 entered the cell and then cushioned onto Ficoll-Pacque (GE Healthcare Life Sciences) to
357 remove residual virus and debris. Infected cells were washed three times with phosphate-
358 buffered saline (PBS), replated in X-VIVO15 at 5 x 10^5 cells/ml, and harvested as indicated in
359 the text. Reactivation in Kasumi-3 cells was induced with 20 nM 12-O-tetradecanoylphorbol-13-
360 acetate (TPA; Fisher) for 48 h in X-VIVO15, while latently infected counterpart cultures were
361 treated with equal volumes of dimethylsulfoxide (DMSO; vehicle) in X-VIVO15.

362 Isolation of CD34$^+$ HPCs is described in detail elsewhere (45). Immediately following
363 isolation, CD34$^+$ HPCs were infected at a multiplicity of 2.0 $TCID_{50}$/cell, as previously described
364 (24, 28, 46), in infection media consisting of IMDM supplemented with 10% BIT9500 serum
365 substitute (Stem Cell Technologies), 2 mM L-glutamine, 20 ng/mL low-density lipoproteins, and
366 50 μM 2-mercaptoethanol. The next day, cultures were washed three times in PBS and replated
367 in 0.4 μm-pore transwells (Corning) over irradiated murine stromal cells in human Long Term
368 Culture Medium (hLTCM, MyeloCult H5100 [Stem Cell Technologies] supplemented with 1 μM
369 hydrocortisone, and 100 U/ml each of penicillin and streptomycin), described in detail in the SI
370 Appendix, SI Materials and Methods.

371 **Multistep Growth Analyses**. Experimental details are included in the SI Appendix, SI Methods.

372 **Extreme Limiting Dilution Assay**

373 Reactivation efficiency in Kasumi-3 cells or CD34$^+$ HPCs was measured by extreme limiting
374 dilution assay (ELDA), as described previously (47). Briefly, Kasumi-3 cells were latently
375 infected for 7 d and then co-cultured with naïve NuFF-1 cells in the presence either of vehicle
376 (DMSO) or 20 nM TPA to maintain latency or induce reactivation, respectively. Infected cells
377 were serially diluted two-fold onto naïve NuFF-1 cells and cultured for 14 d (as described in
378 (45)). For CD34$^+$ HPCs, cells were co-cultured with naïve NuFF-1 cells in a two-fold serial
379 dilution as above, in the presence of hLTCM to maintain latency or reactivation media (RPMI
380 supplemented with 20% FBS, 10 mM HEPES, 1 mM sodium pyruvate, 2 mM $_L$-glutamate, 0.1
381 mM 0.1 mM non-essential amino acids, 100 U/ml each of penicillin and streptomycin with 15
382 ng/ml each of IL-6, G-CSF, GM-CSF and IL-3 [all from R&D Systems]). The production of
383 infectious virus was quantified using viral-expressed mCherry as a marker of infection and
384 ELDA software (bioinf.wehi.edu.au/software/elda/index.html).

385 **Chromatin Immunoprecipitation**. Experimental details are provided in the SI Appendix, SI
386 Methods.

387

388 **Acknowledgments**

389

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392

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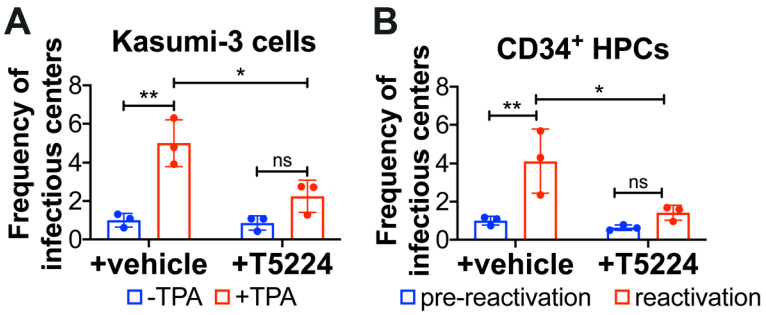
513

514 **Figures**



515

516 **Figure 1. Selective, pharmacological inhibition of c-fos/AP-1 suppresses viral**
517 **reactivation. (A)** Kasumi-3 cells (moi = 1.0) or **(B)** CD34$^+$ HPCs (moi = 2.0) were infected for 7
518 d under latent conditions with WT. Infectious particles were quantified by Extreme Limiting
519 Dilution Analysis (ELDA) with **(A)** DMSO (-TPA, blue bars) or with TPA (+TPA, red bars) or **(B)**
520 hLTCM (pre-reactivation, blue bars) or reactivation media (red bars), in the presence of vehicle
521 (DMSO) or the c-fos inhibitor, T5224 (20 nM). Frequency of infectious centers is plotted as fold-
522 change relative to WT +vehicle under latent conditions. Each data point (circles) is the mean of
523 3 technical replicates. Error bars indicate standard deviation of three biological replicates.
524 Statistical significance was calculated using two-way ANOVA followed by Tukey's post-hoc
525 analysis. *p<0.05, **p<0.01, ns = not significant

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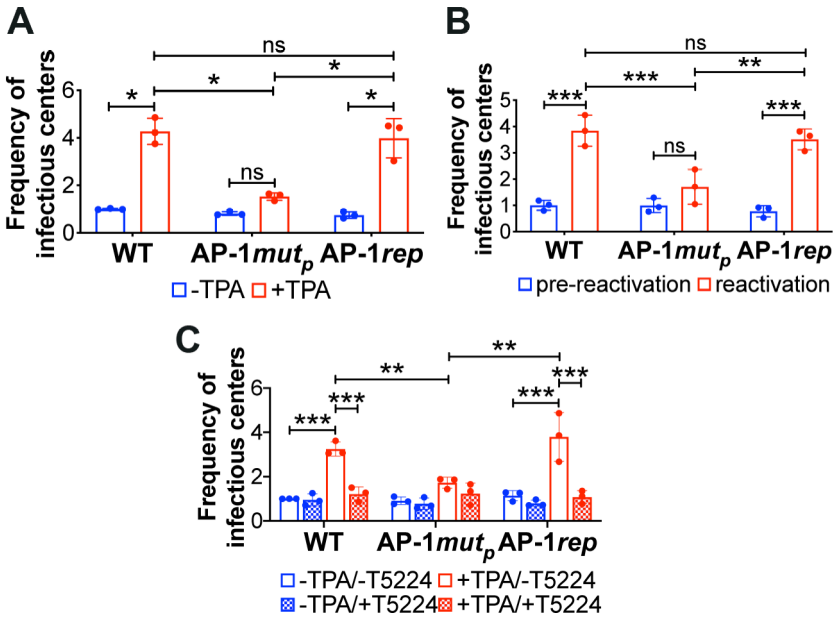

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529

530 **Figure 2. Mutation of the promoter proximal AP-1 binding site in the MIE enhancer**
531 **decreases efficient viral reactivation in Kasumi-3 and primary CD34$^+$ cells. (A,C)** Kasumi-3
532 cells (moi = 1.0) or **(B)** cord blood-derived CD34$^+$ HPCs (moi = 2.0) were infected with the
533 indicated viruses. At 7 dpi, half of each infected population was cultured for an additional 2 d
534 **(A,C)** with vehicle (DMSO; -TPA, blue bars) or TPA (+TPA, red bars) or **(B)** with hLTCM (pre-
535 reactivation, blue bars) or reactivation (red bars) media. **(A,B)** Cells were then co-cultured with
536 naïve NuFF-1 cells to quantify the frequency of infectious centers by ELDA. **(C)** Cells were co-
537 cultured with naïve NuFF-1 cells in the presence of vehicle (DMSO; -T5224, open bars) or the
538 fos inhibitor, T5224 (checked bars), and the frequency of infectious particles was quantified by
539 ELDA. **(A-C)** Each data point (circles) is the mean of 3 technical replicates. Error bars indicate
540 standard deviation of three biological replicates. The statistical significance was calculated using
541 two-way ANOVA followed by Tukey's post-hoc analysis. *p<0.05, ***p*<0.01, ****p*<0.001, and ns
542 = not significant

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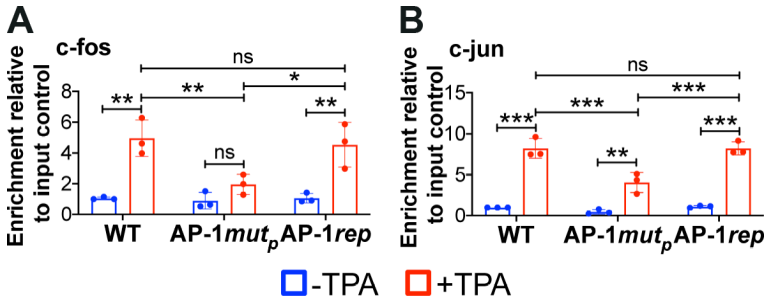
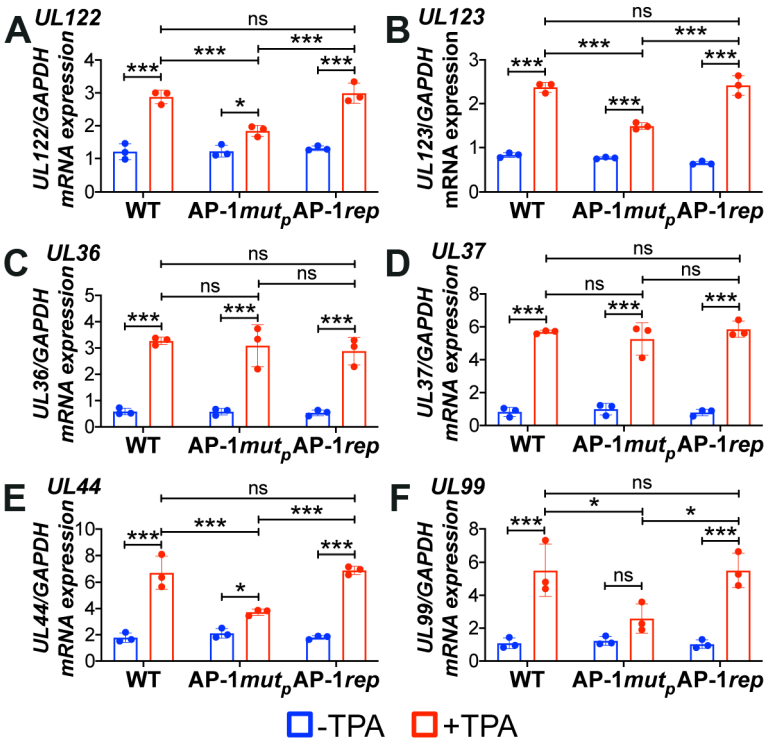


Figure 3. AP-1*mut*$_p$ infection of Kasumi-3 cells results in a significant reduction of AP-1 transcription factor binding to the MIE enhancer in the presence of reactivation stimuli. Kasumi-3 cells were infected (moi = 1.0) with the indicated viruses. At 7 dpi, half of each infected population was cultured for an additional 2 d with vehicle (DMSO; -TPA, blue bars) or TPA (+TPA, red bars). AP-1 transcription factor binding to the MIEP was quantified by ChIP using **(A)** α-fos or **(B)** α-jun antibodies. Co-precipitated MIEP was quantified by qPCR, and data are shown as fold change relative to input. Each data point (circles) is the mean of 3 technical replicates. Error bars indicate standard deviation of three biological replicates, and the statistical significance was calculated using two-way ANOVA followed by Tukey's post-hoc analysis. *p<0.05, **p<0.01, ***p<0.001, and ns = not significant



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558

559 **Figure 4. AP-1*mut*$_p$ infection of Kasumi-3 cells impacts MIEP-driven gene expression, but**
560 **not the transcription of other IE genes.** Kasumi-3 cells were latently infected (moi = 1.0) with
561 the indicated viruses. At 7 dpi, half of each infected population was cultured for an additional 2 d
562 with vehicle (DMSO) to maintain latency (-TPA, blue bars) or with TPA to induce reactivation
563 (red bars). Representative **(A,B)** MIE, **(C,D)** IE, **(E)** E, and **(F)** L genes were assessed by
564 RTqPCR relative to cellular *GAPDH*. Each data point (circles) is the mean of 3 technical
565 replicates. Error bars indicate standard deviation of three biological replicates. The statistical
566 significance was calculated using two-way ANOVA followed by Tukey's post-hoc analysis.
567 *p<0.05, ***p<0.001, and ns = not significant

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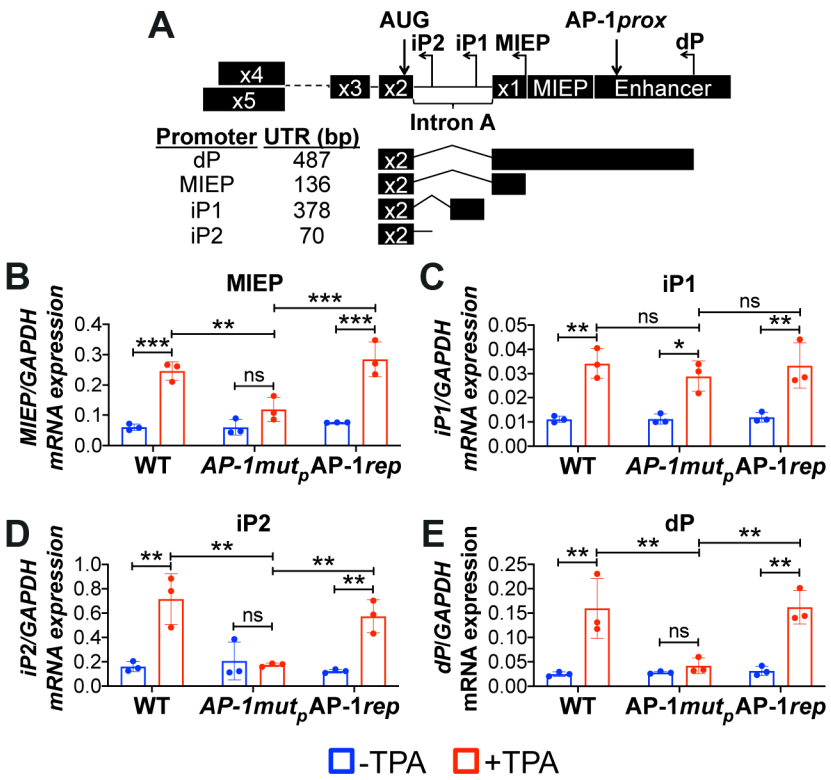

Figure 5. Transcripts driven from the canonical MIEP, iP2, and dP require AP-1 binding following reactivation. Kasumi-3 cells were infected (moi = 1.0) with the indicated viruses. At 7 dpi, half of each infected population was cultured for an additional 2 d with vehicle (DMSO; -TPA, blue bars) or TPA (+TPA, red bars). Transcripts derived from the promoters within the MIE region (not to scale) depicted in **(A)** were assessed: **(B)** MIEP; intronic promoters, **(C)** iP1 and **(D)** iP2; and **(E)** the distal promoter (dP). **(B-E)** Expression of each transcript was measured by RTqPCR and is shown relative to cellular *GAPDH*. Each data point (circles) is the mean of 3 technical replicates, and the error bars indicate standard deviation of three biological replicates. Statistical significance was calculated using two-way ANOVA followed by Tukey's post-hoc analysis. *p<0.05, **p<0.01, ***p<0.001, and ns = not significant