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

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Classification

Biological Sciences, Microbiology

Keywords

HCMV, herpesvirus, AP-1, latency, reactivation

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.

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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 prevent clinically detectable viremia, 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). Reactivation is triggered by differentiation of latently infected myeloid cells, which when combined with immunodeficiency, results in detectable viremia (3, 4) that is treated with antiviral therapies, such as valganciclovir and foscarnet (5). 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 (6, 7). 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 (8). A critical determinant in the establishment and maintenance of HCMV latency is repression of the major immediate early promoter (MIEP). The MIEP is a

76 powerful promoter, which drives the expression of the major immediate early (MIE) genes,
77 *UL122* and *UL123* that encode IE2 and IE1, respectively. These MIE proteins transactivate
78 other sites of E gene expression, which in turn facilitate the lytic life cycle. As such, the MIE
79 locus is significantly repressed during viral latency (9-12). This silencing is mediated, at least in
80 part, by the recruitment of both repressive transcription factors and chromatin modifiers to the
81 MIE enhancer/promoter region. During reactivation from latency, these repressive factors are
82 overcome, resulting in de-repression of the MIE enhancer/promoter and expression of the MIE-
83 driven genes. This likely requires a multifaceted process that exchanges pro-latency factors for
84 transcription factors and chromatin remodeling favoring the re-expression of IE1 and IE2 (3). It
85 is also clear that additional promoters within the MIE region drive transcription of mRNAs with
86 differing 5' untranslated region (UTR) lengths, all of which can encode full length IE1 and IE2
87 proteins. Two of these non-canonical transcripts are derived from promoters within intron A of
88 the MIE region, termed iP1 and iP2 (13). Interestingly, recent work from Collins-McMillen, et al.
89 demonstrated iP2-derived transcripts are the most abundant during both latency and
90 reactivation. Furthermore, transactivation of both iP1 and iP2 are required for efficient viral
91 reactivation in primary $CD34^+$ hematopoietic progenitor cells (HPCs) (14), though the regulation
92 of these newly identified promoters remains unknown. These findings highlight the complexity of
93 the transcriptional control of the MIE locus.

94 Work from many investigators implicates multiple factors in de-repressing the MIE locus
95 during reactivation. These include: src family kinase activation of extracellular signal-regulated
96 kinase/mitogen-activated protein kinase (ERK/MAPK; ref. (15)), cyclic AMP (cAMP) activation
97 (16), cAMP response element-binding protein (CREB) phosphorylation (17), epidermal growth
98 factor receptor (EGFR) activation of phosphoinositide 3-kinase (PI3K; ref. (18)), facilitates
99 chromatin transcription (FACT)-mediated transactivation (19), mammalian target of rapamycin
100 (mTOR)-modulated activation of KRAB-associated protein-1 (KAP1; ref. (20)), and protein
101 kinase A (PKA)-CREB-TORC2 transactivation (21). Although these results summarize findings
102 from a variety of latency model systems, they underscore the idea that reactivation likely
103 requires concomitant signals from multiple pathways and factors to de-repress the MIE locus,
104 triggering epigenetic changes as well as the recruitment of transcription factors that
105 transactivate this region.

106 We recently showed *US28*, an HCMV encoded G-protein coupled receptor (GPCR),
107 attenuates cellular fos (c-fos) signaling to maintain latency (22). Dimerization of c-fos and c-jun
108 form the transcription factor, activator protein-1 (AP-1), which binds and transactivates the MIEP

109 in luciferase reporter assays (23). US28 protein (pUS28) suppression of phosphorylated c-fos
110 prevents AP-1 from binding the MIE enhancer and activating the MIE locus during latent
111 infection of Kasumi-3 cells (a $CD34^+$ cell line that supports latency and viral reactivation) and
112 primary *ex vivo* $CD34^+$ hematopoietic progenitor cells (HPCs) (22). The MIE enhancer region
113 contains two AP-1 binding sites: the promoter proximal binding site has a canonical AP-1
114 binding motif, while the promoter distal binding site has a non-consensus sequence (23-25).
115 Disruption of both binding sites together does not impact lytic replication in fibroblasts or
116 epithelial cells, nor does it affect virulence in a chimeric murine CMV infection model (23),
117 suggesting AP-1 recruitment to the MIEP is dispensable for lytic infection.
118 Our previous data revealed suppressing AP-1 activity is necessary for maintaining
119 HCMV latency (22), and thus we hypothesized AP-1 recruitment to the MIEP is important for
120 efficient reactivation from latency in hematopoietic cells. Herein, we show pharmacological
121 inhibition of AP-1 in the presence of reactivation stimuli decreases reactivation efficiency.
122 Additionally, mutation of the promoter proximal AP-1 binding site attenuates AP-1 recruitment to
123 the MIE enhancer and impairs reactivation following stimuli. Finally, our data reveal AP-1
124 recruitment to the promoter proximal site in the MIE enhancer during reactivation is required to
125 stimulate the expression of MIE-driven genes originating from the canonical MIEP, iP2, and the
126 distal promoter (dP), but not other IE genes or iP1-driven transcripts. Taken together, our results
127 show AP-1 transactivation of the MIE enhancer/promoter is an important factor for efficient
128 HCMV reactivation from latency.
129

130 **Results**

131 **Pharmacological inhibition of c-fos suppresses viral reactivation in Kasumi-3 cells**
132 We previously showed HCMV suppresses AP-1 activation to maintain latency (22), which led us
133 to posit the requirement for this transcription factor's activation during reactivation from latency.
134 To test the impact AP-1 signaling has on reactivation, we infected Kasumi-3 cells with
135 TB40/E*mCherry* (WT) and cultured the cells in conditions favoring latency for 7 days (d).
136 Cultures were then treated with vehicle (dimethylsulfoxide, DMSO) to maintain latency or
137 tetradecanoyl phorbol acetate (TPA) to induce reactivation for an additional 2 d in the presence
138 or absence of the c-fos inhibitor, T-5224, at a concentration we previously showed does not
139 impact cell viability (22). We assessed the ability of each infection to reactivate by co-culturing
140 the infected Kasumi-3 cells with naïve fibroblasts and quantified infectious virus production by

141 Extreme Limiting Dilution Analysis (ELDA). As expected, TPA treatment of infected Kasumi-3
142 cells induced reactivation, as measured by an increase in virus production (26). However,
143 treatment with T-5224 attenuated virus production in TPA-treated cells (Fig. 1A). We next
144 confirmed this finding using cord blood-derived, *ex vivo* cultured $CD34^+$ HPCs, a natural site of
145 HCMV latency. We infected these primary cells with WT virus for 7 d under conditions favoring
146 latency, after which we cultured a portion of the infected cells in media that promotes
147 reactivation (27) either with or without T-5224. Similar to Kasumi-3 cells, our findings reveal
148 treatment with T-5224 reduced virus production in $CD34^+$ HPCs treated with reactivation stimuli
149 (Fig. 1B). Together, these results indicate AP-1 activation is important for efficient reactivation
150 from latency.
151

152 **Mutation of the promoter proximal AP-1 binding site in the MIE enhancer impairs viral**
153 **reactivation in hematopoietic cells**

154 Based on our findings above, we next asked if AP-1 recruitment to the MIE enhancer aids in de-
155 repressing the MIE locus during HCMV reactivation. To begin to investigate the contribution of
156 AP-1 to this process, we generated a recombinant virus in the bacterial artificial chromosome
157 (BAC)-derived, clinical isolate TB40/E (28), which we previously engineered to express mCherry
158 (TB40/E*mCherry*) (29). We disrupted the promoter proximal AP-1 binding site to generate
159 TB40/E*mCherry*-proximal-AP-1*mutant* (AP-1mut_p). Additionally, to ensure we did not introduce
160 off-site mutations during the recombineering process, we restored the mutated MIE
161 enhancer/promoter region to its wild type sequence to generate the repair virus,
162 TB40/E*mCherry*-proximal-AP-1*repair* (AP-1rep_p) (SI Appendix, Fig. S2). Consistent with
163 previous findings (23), we confirmed AP-1mut_p and AP-1rep_p display WT growth phenotypes in
164 fibroblasts (SI Appendix, Fig. S3A). Similarly, both the mutant and repair viruses displayed WT
165 growth following lytic infection of epithelial cells (SI Appendix, Fig. S3B). Together, these results
166 suggest AP-1 recruitment to the promoter proximal site is not required for efficient lytic
167 replication.

168 To determine the contribution of the AP-1 promoter proximal binding site during
169 reactivation from latency, we infected Kasumi-3 cells with WT, AP-1mut_p, or AP-1rep_p for 7 d
170 under conditions favoring latency, after which we performed ELDA in the presence of vehicle or
171 TPA treatment. We found AP-1mut_p-infected Kasumi-3 cells failed to efficiently reactivate to WT
172 or AP-1rep_p levels (Fig. 2A). Importantly, AP-1mut_p-infected cells maintain comparable levels of
173 the viral genome compared to WT- or AP-1rep_p-infected cultures (SI Appendix, Fig. S5),

174 suggesting these infected cultures harbor latent virus. Similarly, the latency-associated gene,
175 *UL138*, was highly expressed relative to *UL123* in all infected cultures, consistent with latent
176 infection (SI Appendix, Fig. S6). Treatment of each infected culture with TPA resulted in a
177 decrease in the ratio of *UL138*/*UL123* (SI Appendix, Fig. S6), consistent with viral reactivation
178 (22, 26, 30). The ratio of *UL138*:*UL123* neared 1 in the WT- and AP-1rep_p-infected cells
179 following TPA treatment, which was significantly lower than the ratio of these transcripts in the
180 AP-1mut_p-infected counterpart cultures (SI Appendix, Fig. S6), suggesting inefficient reactivation
181 of the mutant virus. Consistent with these observations, AP-1mut_p-infected primary $CD34^+$
182 HPCs produces significantly less infectious virus than either WT or AP-1rep_p when stimulated to
183 reactivate (Fig. 2B). Together, these data suggest AP-1mut_p-infected hematopoietic cells
184 undergo latency, but fail to efficiently reactivate to WT levels following stimuli.

185 Finally, to distinguish between the relative contributions of AP-1 activity and the
186 requirement for the AP-1 promoter proximal binding site during reactivation from latency, we
187 evaluated viral reactivation in the presence or absence of the c-fos inhibitor, T-5224. To this
188 end, we infected Kasumi-3 cells with WT, AP-1mut_p, or AP-1rep_p for 7 d under conditions
189 favoring latency. We then quantified infectious virus production by ELDA in the presence or
190 absence of TPA and/or T-5224. In the presence of T-5224, WT- and AP-1rep_p-infected cells fail
191 to efficiently reactivate virus, resulting in virion production similar to AP-1mut_p-infected cells in
192 the absence of T-5224 treatment (Fig. 2C). Further, T-5224 did not further impair reactivation of
193 the AP-1mut_p virus. Taken together, these results demonstrate the importance of AP-1
194 activation and the requirement for the promoter proximal site of the MIE enhancer in promoting
195 HCMV reactivation.

196

197 **Recruitment of the AP-1 transcription factor to the MIE enhancer is significantly**
198 **diminished following reactivation stimulus of AP-1mut_p-infected Kasumi-3 cells**

199 To test our hypothesis that AP-1 binds to the MIE enhancer, we first evaluated the recruitment
200 of the AP-1 subunits, c-fos and c-jun, to the enhancer region. We latently infected Kasumi-3
201 cells with WT, AP-1mut_p, or AP-1rep_p for 7 d and then treated a portion of the infected cultures
202 for an additional 2 d with TPA treatment to induce reactivation. We then harvested the cultures
203 and performed chromatin immunoprecipitation (ChIP) assays to measure binding of c-fos and c-
204 jun to the MIE enhancer. Our results show both c-fos and c-jun bind the MIE enhancer following
205 TPA treatment of WT, latently infected Kasumi-3 cells, although disruption of the promoter
206 proximal AP-1 site reduced recruitment of each AP-1 subunit (Fig. 3). Importantly, AP-1mut_p

207 infection does not impact c-fos or c-jun transcript levels, which are comparable to those in WT-
208 and AP-1*rep*$_p$-infected cells both prior to and following TPA treatment (SI Appendix, Fig. S7).
209 Further, consistent with previous findings (22, 31), c-fos and c-jun mRNA is attenuated during
210 latent infection relative to conditions favoring reactivation (SI Appendix, Fig. S7). While both c-
211 fos and c-jun recruitment is significantly attenuated in the AP-1*mut*$_p$-infected Kasumi-3 cells, our
212 data reveal neither AP-1 subunit is completely devoid of binding. This suggests AP-1 also binds
213 the promoter distal, non-consensus AP-1 binding site in the MIE enhancer region. To address
214 this, we generated the AP-1 promoter distal mutant, termed AP-1*mut*$_d$, and its match repair
215 virus, AP-1*rep*$_d$. We also generated a viral mutant in which we mutated both proximal and distal
216 AP-1 binding sites in a single background, termed AP-1*mut*$_{pd}$, as well as a repair virus, in which
217 we returned both mutated sequences to wild type, AP-1*rep*$_{pd}$ (SI Appendix, Fig. S2). Each of
218 these viruses replicated to wild type titers in primary fibroblasts (SI Appendix, Fig. S8A),
219 indicating AP-1 binding to the MIE enhancer is not required for efficient lytic replication.
220 However, viral reactivation was significantly impaired in AP-1*mut*$_{pd}$-infected Kasumi-3 cells
221 treated with TPA, which was not significantly different when compared to cells infected with AP-
222 1*mut*$_p$, whereas AP-1*mut*$_d$-infected Kasumi-3 cells reactivated to WT levels following TPA
223 treatment (SI Appendix, Fig. 8B). Further, both c-fos and c-jun subunits of AP-1 bound the MIE
224 enhancer in AP-1*mut*$_d$-infected, but not AP-1*mut*$_{pd}$-infected Kasumi-3 cells treated with TPA (SI
225 Appendix, Fig. 8C and D). Our data collectively indicate the promoter distal AP-1 binding site
226 does not play a significant role in reactivation from latency, whereas deletion of the promoter
227 proximal site alone significantly impacts efficient reactivation following stimuli (Fig. 2A and B, SI
228 Appendix, Fig. 8B), despite detectable promoter distal site binding of AP-1 (Fig. 3, SI Appendix,
229 Fig. 8C and D).
230

231 **AP-1 recruitment to the MIE enhancer during reactivation initiates MIE-driven gene**
232 **expression, but not the transcription of other IE genes**

233 Together, our data reveal AP-1 recruitment to the promoter proximal site in the MIE enhancer is
234 an important determinant for efficient viral reactivation. Thus, we hypothesized this transcription
235 factor is critical for de-repression of *UL122* and *UL123* expression. In line with this, we posited
236 the recruitment of AP-1 to the MIE enhancer would not impact other IE genes. To this end, we
237 latently infected Kasumi-3 cells with WT, AP-1*mut*$_p$, or AP-1*rep*$_p$ for 7 d, after which we treated a
238 portion of the cells with TPA for an additional 2 d to stimulate reactivation. We then harvested
239 total RNA from the infected cultures and analyzed HCMV gene expression by RT-qPCR. We

240 assessed the MIE-driven transcripts *UL122* and *UL123*, representative non-MIE-derived IE
241 genes (*UL36*, *UL37*), as well as a representative early (E; *UL44*) and late (L; *UL99*) transcripts.
242 AP-1mut_p-infected Kasumi-3 cells treated with TPA displayed attenuated *UL122* and *UL123*
243 transcription compared to the WT- or AP-1rep_p-infected cultures (Fig. 4A and B). As expected,
244 this decreased E (*UL44*, Fig. 4E) and L (*UL99*, Fig. 4F) transcript abundance, whose
245 transactivation is dependent upon MIE-driven transcription and their subsequent translation
246 (32). However, transcription of *UL36* and *UL37*, whose expression is not regulated by the MIE
247 enhancer, was unaffected by the mutation of the promoter proximal AP-1 site (Fig. 4C and D).
248 Taken together, these data suggest AP-1 recruitment to the promoter proximal site within the
249 MIE enhancer is required for the expression of MIE-driven transcripts and efficient viral
250 reactivation.

251 Thus far, our findings indicate disruption of promoter proximal AP-1 binding to the MIE
252 enhancer impairs de-repression of MIE transcript expression. However, IE1 and IE2 are
253 translated from multiple transcripts originating from the canonical MIEP, as well as alternative
254 transcription start sites (TSSs) within the MIE region. While these alternative transcripts differ in
255 their 5'UTR, each encodes full-length IE1 or IE2 protein (13). More recently, Collins-McMillen et
256 al. found two of these alternative transcripts derived from promoters within intron A, iP1 and iP2,
257 are important for reactivation from latency (14). However, the contribution of transcription factor
258 binding on the activity of these alternative promoters during reactivation is unknown. To this
259 end, we infected Kasumi-3 cells as above and analyzed expression of MIE mRNAs by RT-
260 qPCR, using primers specific for the transcripts derived from the dP, canonical MIEP, or the
261 internal promoters, iP1 and iP2 (Fig. 5A). Following TPA-induced reactivation of WT-infected
262 cultures, transcripts originating from each promoter increased (Fig. 5B-E), concomitant with
263 *UL122* (Fig. 4A) and *UL123* (Fig. 4B) transcription. These findings are consistent with previous
264 findings using the THP-1 culture system (14). However, AP-1mut_p-infected Kasumi-3 cells
265 displayed impaired MIEP-, iP2-, and dP-derived transcripts compared to cultures infected with
266 either WT or AP-1rep_p (Fig. 5B, D, and E, respectively). In contrast to these MIE-derived
267 transcripts, mRNA driven from the iP1 promoter was expressed to similar levels following
268 reactivation when compared to WT- or AP-1rep_p-infected cultures (Fig. 5C), suggesting iP1-
269 driven transcription is independent of AP-1 binding to the promoter proximal site in the MIE
270 enhancer. Taken together, these data suggest AP-1 binding to the MIE enhancer facilitates the
271 expression of MIEP-, iP2-, and dP-derived transcripts, while iP1-driven transcription remains
272 independent of AP-1 recruitment to this region.

273

274 **Discussion**

275 HCMV reactivation is a multifaceted process, requiring de-repression of *UL122* and *UL123*
276 transcription, changes in transcription factor binding, as well as changes in chromatin structure
277 to promote transcription (3). In this study, we found AP-1 binding to the MIE enhancer is critical
278 for the re-expression of both *UL122* and *UL123*, as well as HCMV reactivation.
279 Pharmacological inhibition of c-fos, a component of AP-1, significantly reduced the reactivation
280 efficiency of WT-infected hematopoietic cells (Fig. 1). Similarly, disruption of the promoter
281 proximal AP-1 binding site within the MIE enhancer attenuated HCMV reactivation (Fig. 2). Our
282 data demonstrate AP-1 is recruited to the MIE enhancer following reactivation of WT virus, and
283 mutation of the promoter proximal AP-1 binding site significantly decreases AP-1 binding (Fig.
284 3). Further, while the MIE enhancer has two AP-1 binding sites, our data emphasize the
285 promoter proximal site is critical for viral reactivation (Fig. 2, 3, SI Appendix Fig. S8). AP-1
286 recruitment to the MIE enhancer aids in the transactivation of the MIE-driven IE genes, *UL122*
287 and *UL123*, while IE genes not derived from the MIE region, including *UL36* and *UL37*, remain
288 unaffected (Fig. 4). Importantly, AP-1 binding to its promoter proximal site in the MIE enhancer
289 activates transcription from the canonical MIEP, as well as the alternative iP2 and dP
290 promoters, while iP1 activity is AP-1-independent (Fig. 5). Together, our results reveal a
291 mechanism by which the AP-1 transcription factor binds the MIE enhancer to activate multiple
292 promoters in the MIE locus, thereby contributing to successful reactivation of HCMV from
293 latency.

294 While viral reactivation is likely multifactorial, it is clear c-fos and c-jun are critical
295 components of this process. Our previous findings showed pUS28 attenuates c-fos expression
296 and activation, concomitant with reduced AP-1 recruitment to the MIE enhancer during latency
297 (22). Additionally, we have shown c-jun expression is significantly suppressed during HCMV
298 latency (31), although this is not contingent upon pUS28 expression or function (22). These
299 findings suggest HCMV has adapted independent mechanisms to attenuate the two subunits
300 comprising AP-1 to prevent its binding to the MIE enhancer during latency.

301 Our findings also suggest HCMV evolved sites for AP-1 recruitment to the MIE enhancer
302 region for viral reactivation, although this association is not required for efficient lytic replication.
303 This is not unprecedented, as similar mechanisms were described for the CRE response
304 elements (17, 33). Consistent with this, several groups have shown AP-1 binds to and activates

305 the MIE enhancer/promoter in *in vitro* expression assays (23, 33, 34). In agreement with our
306 results herein, AP-1 is recruited to the MIE enhancer in a murine model of allograft-induced
307 murine CMV (MCMV) reactivation (35). Furthermore, work from Isern, et al, revealed disruption
308 of both AP-1 binding sites within the MIE enhancer region had no effect on HCMV lytic
309 replication in fibroblast and epithelial cells (23), consistent with our current findings. Additionally,
310 Isern, et al. generated a chimeric MCMV, replacing the native murine MIE enhancer with the
311 HCMV enhancer region, and then disrupted both AP-1 binding sites. Using this chimeric
312 recombinant in either lung or spleen explants of neonatal mice, they found no difference in virus
313 reactivation relative to infection with a chimeric virus containing wild type AP-1 binding sites
314 (23). While this work revealed no role for the promoter proximal AP-1 binding site, there are
315 significant differences in the experimental models we employed herein and the chimeric MCMV-
316 infected mouse models used in the previously published study (23).

317 In addition to HCMV, other herpesviruses modulate AP-1 during infection. The Epstein
318 Barr virus (EBV) protein, BGLF2, and the Kaposi's sarcoma-associated herpesvirus (KSHV)-
319 encoded ORF45 both activate the AP-1 signaling pathway to promote viral gene expression,
320 replication, and survival (36, 37). EBV also encodes an AP-1 homolog, BZLF1, which, like AP-1,
321 supports resting B cell proliferation and binds methylated EBV promoters critical for reactivation
322 (38). Murine gammaherpesvirus 68 (MHV68) expresses the microRNA mghv-miR-M1-1, which
323 downregulates c-jun and subsequent AP-1 activation, resulting in the suppression of viral lytic
324 replication (39). Together, these results show controlling AP-1 is a common strategy among
325 herpesviruses, suggesting targeting this transcription factor could prove beneficial for
326 suppressing reactivation and subsequent disease associated with a variety of herpesviruses.

327 Recently published findings from Collins-McMillen, et al. detailed the requirement for
328 iP1- and iP2-derived transcripts for viral reactivation (14). Indeed, consistent with their findings,
329 we also found the most abundant transcripts are derived from the iP2 alternative promoter, both
330 before and after reactivation, although MIEP-derived transcripts were more abundant than those
331 transcribed from iP1 in our system (Fig. 5). While our overall goal was not to discern differences
332 in abundances, this difference could be due to the systems used: we measured these
333 transcripts in Kasumi-3 cells, while Collins-McMillen determined transcript abundance over time
334 in THP-1 cells (14). Nonetheless, our findings build upon these prior observations and begin to
335 unravel the likely complex web of transcription factors that regulate the iP1 and iP2 promoters.
336 We found AP-1 recruitment to the promoter proximal site within the MIE enhancer drives
337 transcription from the MIEP, iP2, and dP (Fig. 5B, D, and E, respectively). However,

338 transcription from iP1 is independent of AP-1 recruitment to the promoter proximal site within
339 the MIE enhancer, as the AP-1*mut*$_p$-infected cells express iP1-driven transcripts to levels similar
340 to those we observed in WT-infected cells (Fig. 5C). This finding suggests iP1-driven
341 transcription alone is not sufficient to drive reactivation, as AP-1*mut*$_p$-infected cells fail to
342 efficiently produce infectious virus following the addition of reactivation stimuli (Fig. 2). This
343 result also suggests additional transcription factors or chromatin remodeling proteins may
344 alternatively control iP1 activity, such as FOXO3A (personal communication, Nat Moorman),
345 further highlighting the complexity in the regulation of transcription in MIE locus.

346 How is AP-1 activated in response to cues that reactivate HCMV? Elucidating the
347 precise biological mechanism could indeed reveal potential therapeutic targets one could exploit
348 to prevent viral reactivation. Two pathways that activate AP-1, Src-ERK (40) and PI3K/Akt (41),
349 are already implicated in HCMV reactivation (15, 18) and are therefore potential candidates for
350 further investigation. As a potent signaling molecule, US28 is an attractive, latently expressed
351 viral protein capable of modulating cellular signaling pathways such as these. While our
352 previous data showed US28 attenuates c-fos expression and activity as well as AP-1 binding to
353 the MIE enhancer during latency (22), it remains unknown how US28 signaling is modified to
354 favor reactivation rather than latent conditions. It is attractive to hypothesize an additional viral
355 or cellular protein is triggered to alter US28's behavior following reactivation stimuli, though
356 additional work is needed to realize such mechanistic nuances. It is also possible AP-1 recruits
357 other factors, such as histone acetyltransferases (HATs) (42) to facilitate chromatin remodeling
358 and/or other transcription factors (e.g. CREB and NFκB), all of which would contribute to de-
359 repression of MIE promoters (3). In reporter assays, pp71 transactivates promoters containing
360 AP-1 or CREB in human fibroblasts (43). While pp71, in conjunction with cellular DAXX and
361 ATRX, aids in silencing the MIE enhancer/promoter via repressive heterochromatic marks and
362 histone deposition (44), whether pp71 transactivates the MIE enhancer/promoter during
363 reactivation via AP-1 and or CREB, remains elusive. Finally, c-fos and c-jun functional activity
364 contribute to myeloid differentiation and function in response to pro-inflammatory stimuli (45).
365 These possibilities present numerous avenues for future research aimed at understanding the
366 underlying biological mechanisms regulating AP-1 activation and its transactivation of the MIE
367 enhancer/promoter region.

368 Our findings detailed herein reveal AP-1 activation is critical for HCMV reactivation. AP-1
369 recruitment to its promoter proximal site within the MIE enhancer activates multiple MIE
370 promoters, including the dP, MIEP, and iP2, to drive MIE transcription, which overall facilitates

371 reactivation. Herein, we provide a novel mechanism underlying MIE de-repression during HCMV
372 reactivation, further illuminating the molecular details essential for understanding HCMV latency
373 and reactivation that will lead to the development of novel therapies to prevent viral reactivation
374 in vulnerable patients.
375
376 **Methods**

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

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

382 **Infection of Kasumi-3 and CD34$^+$ Cells**. Kasumi-3 cells were infected as described previously
383 (22, 26, 30). Briefly, cells were serum-starved in X-VIVO15 (Lonza) 48 hours (h) before infection
384 and then infected at a multiplicity of 1.0 50% tissue culture infectious dose ($TCID_{50}$)/cell by
385 centrifugal enhancement (1,000 × *g*, 35 minutes [min], room temperature) at 5 x 10^5 cells/ml in
386 X-VIVO15. The following day, cells were treated with trypsin to remove any virus that had not
387 entered the cell and then cushioned onto Ficoll-Pacque (GE Healthcare Life Sciences) to
388 remove residual virus and debris. Infected cells were washed three times with phosphate-
389 buffered saline (PBS), replated in X-VIVO15 at 5 x 10^5 cells/ml, and harvested as indicated in
390 the text. Reactivation in Kasumi-3 cells was induced with 20 nM 12-O-tetradecanoylphorbol-13-
391 acetate (TPA; Fisher) for 48 h in X-VIVO15, while latently infected counterpart cultures were
392 treated with equal volumes of dimethylsulfoxide (DMSO; vehicle) in X-VIVO15.

393 Isolation of CD34$^+$ HPCs is described in detail elsewhere (27). Immediately following
394 isolation, CD34$^+$ HPCs were infected at a multiplicity of 2.0 $TCID_{50}$/cell, as previously described
395 (22, 26, 30, 46), in infection media consisting of IMDM supplemented with 10% BIT9500 serum
396 substitute (Stem Cell Technologies), 2 mM L-glutamine, 20 ng/mL low-density lipoproteins, and
397 50 μM 2-mercaptoethanol. The next day, cultures were washed three times in PBS and replated
398 in 0.4 μm-pore transwells (Corning) over irradiated murine stromal cells in human Long Term
399 Culture Medium (hLTCM, MyeloCult H5100 [Stem Cell Technologies] supplemented with 1 μM
400 hydrocortisone, and 100 U/ml each of penicillin and streptomycin), described in detail in the SI
401 Appendix, SI Materials and Methods.

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

403 **Extreme Limiting Dilution Assay**. Reactivation efficiency in Kasumi-3 cells or $CD34^+$ HPCs
404 was measured by extreme limiting dilution assay (ELDA), as described previously (27). Briefly,
405 Kasumi-3 cells were latently infected for 7 d and then co-cultured with naïve NuFF-1 cells in the
406 presence either of vehicle (DMSO) or 20 nM TPA to maintain latency or induce reactivation,
407 respectively. Infected cells were serially diluted two-fold onto naïve NuFF-1 cells and cultured
408 for 14 d (as described in (27)). For $CD34^+$ HPCs, cells were co-cultured with naïve NuFF-1 cells
409 in a two-fold serial dilution as above, in the presence of hLTCM to maintain latency or
410 reactivation media (RPMI supplemented with 20% FBS, 10 mM HEPES, 1 mM sodium
411 pyruvate, 2 mM $_L$-glutamate, 0.1 mM 0.1 mM non-essential amino acids, 100 U/ml each of
412 penicillin and streptomycin with 15 ng/ml each of IL-6, G-CSF, GM-CSF and IL-3 [all from R&D
413 Systems]). The production of infectious virus was quantified using viral-expressed mCherry as a
414 marker of infection and ELDA software (bioinf.wehi.edu.au/software/elda/index.html).

415 **Chromatin Immunoprecipitation**. Experimental details are provided in the SI Appendix, SI
416 Methods.

417

418 **Acknowledgments**

419

420 This work was supported by the American Heart Association Scientist Development Grant
421 15SDG23000029 (to C.M.O'C.) and Cleveland Clinic funding (to C.M.O'C.).

422

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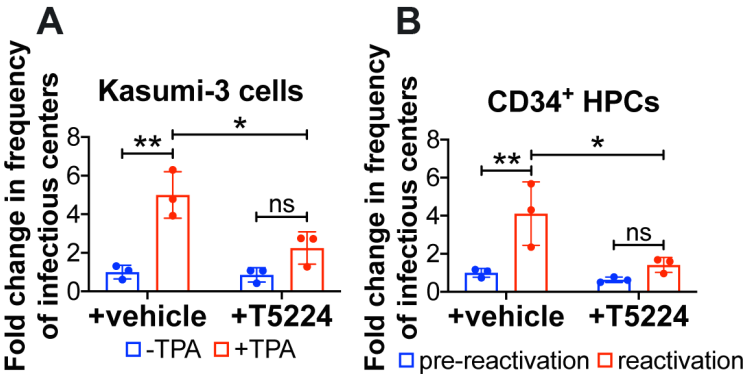
539

540 **Figures**



541

542 **Figure 1. Selective, pharmacological inhibition of c-fos/AP-1 suppresses viral**
543 **reactivation. (A)** Kasumi-3 cells (moi = 1.0) or **(B)** CD34$^+$ HPCs (moi = 2.0) were infected for 7
544 d under latent conditions with WT. Infectious particles were quantified by Extreme Limiting
545 Dilution Analysis (ELDA) with **(A)** DMSO (-TPA, blue bars) or with TPA (+TPA, red bars) or **(B)**
546 hLTCM (pre-reactivation, blue bars) or reactivation media (red bars), in the presence of vehicle
547 (DMSO) or the c-fos inhibitor, T5224 (20 nM). Frequency of infectious centers is plotted as fold-
548 change relative to WT +vehicle under latent conditions. Each data point (circles) is the mean of
549 3 technical replicates. Error bars indicate standard deviation of three biological replicates, each
550 of which is shown in SI Appendix, Fig. S1. Statistical significance was calculated using two-way
551 ANOVA followed by Tukey's post-hoc analysis. *p<0.05, **p<0.01, ns = not significant

552

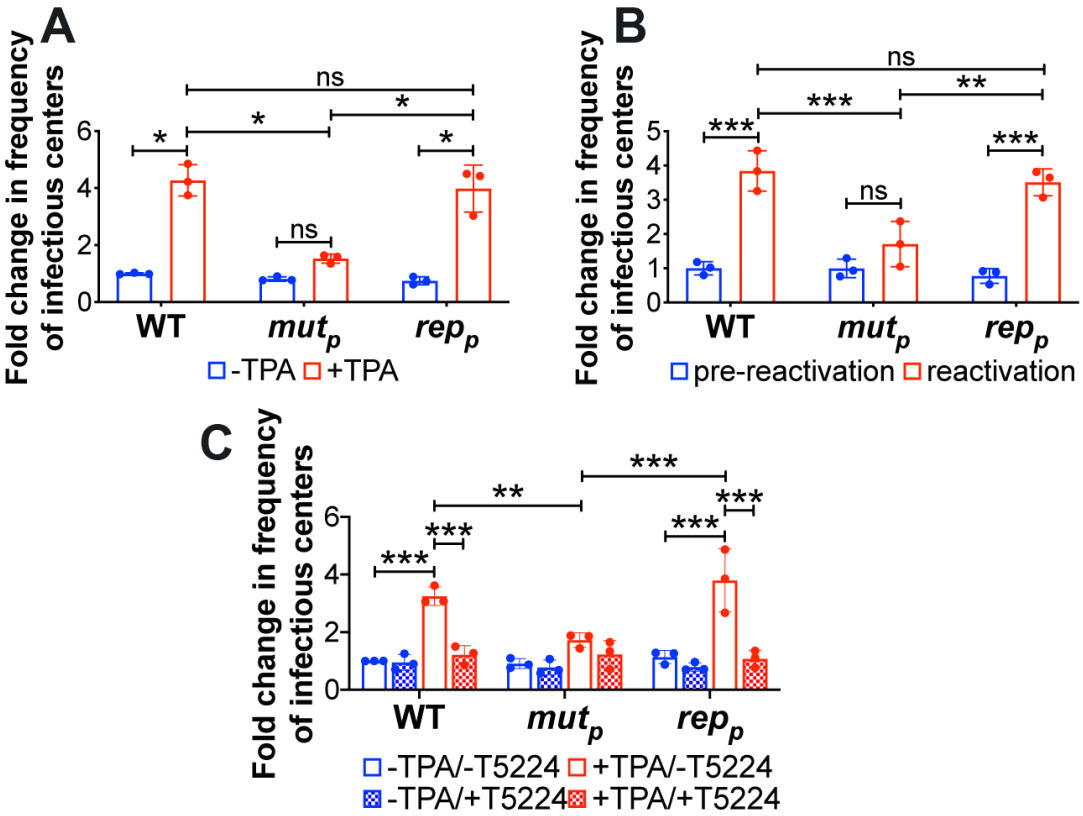


Figure 2. Mutation of the promoter proximal AP-1 binding site in the MIE enhancer decreases efficient viral reactivation in Kasumi-3 and primary CD34$^+$ cells. (A,C) Kasumi-3 cells (moi = 1.0) or **(B)** cord blood-derived CD34$^+$ HPCs (moi = 2.0) were infected with the indicated viruses. At 7 dpi, half of each infected population was cultured for an additional 2 d **(A,C)** with vehicle (DMSO; -TPA, blue bars) or TPA (+TPA, red bars) or **(B)** with hLTCM (pre-reactivation, blue bars) or reactivation (red bars) media. **(A,B)** Cells were then co-cultured with naïve NuFF-1 cells to quantify the frequency of infectious centers by ELDA. **(C)** Cells were co-cultured with naïve NuFF-1 cells in the presence of vehicle (DMSO; -T5224, open bars) or the fos inhibitor, T5224 (checked bars), and the frequency of infectious particles was quantified by ELDA. **(A-C)** Each data point (circles) is the mean of 3 technical replicates. Error bars indicate standard deviation of three biological replicates, each of which is shown in SI Appendix, Fig. S4. Data is presented as fold-change relative to WT **(A)** -TPA, **(B)** pre-reactivation, or **(C)** -TPA/-T5224. 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. Abbreviations: AP-1*mut$_p$* (*mut$_p$*), AP-1*rep$_p$* (*rep$_p$*).

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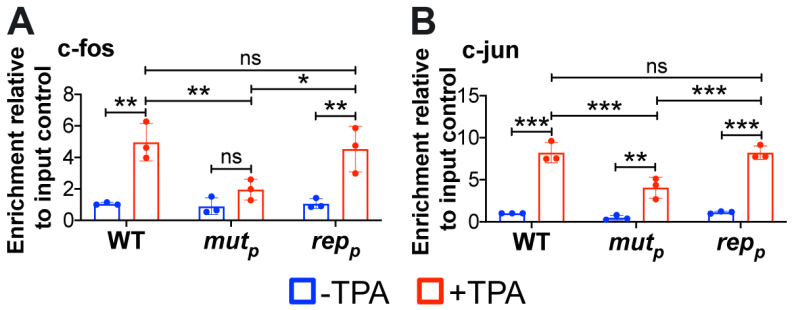

571

572 **Figure 3. AP-1*mut*$_p$ infection of Kasumi-3 cells results in a significant reduction of AP-1**
573 **transcription factor binding to the MIE enhancer in the presence of reactivation stimuli.**
574 Kasumi-3 cells were infected (moi = 1.0) with the indicated viruses. At 7 dpi, half of each
575 infected population was cultured for an additional 2 d with vehicle (DMSO; -TPA, blue bars) or
576 TPA (+TPA, red bars). AP-1 transcription factor binding to the MIEP was quantified by ChIP
577 using **(A)** α-fos or **(B)** α-jun antibodies. Co-precipitated MIEP was quantified by qPCR, and data
578 are shown as fold change relative to input. Each data point (circles) is the mean of 3 technical
579 replicates. Error bars indicate standard deviation of three biological replicates, and the statistical
580 significance was calculated using two-way ANOVA followed by Tukey's post-hoc analysis.
581 *p<0.05, **p<0.01, ***p<0.001, and ns = not significant. Abbreviations: AP-1*mut*$_p$ (*mut*$_p$), AP-
582 1*rep*$_p$ (*rep*$_p$).

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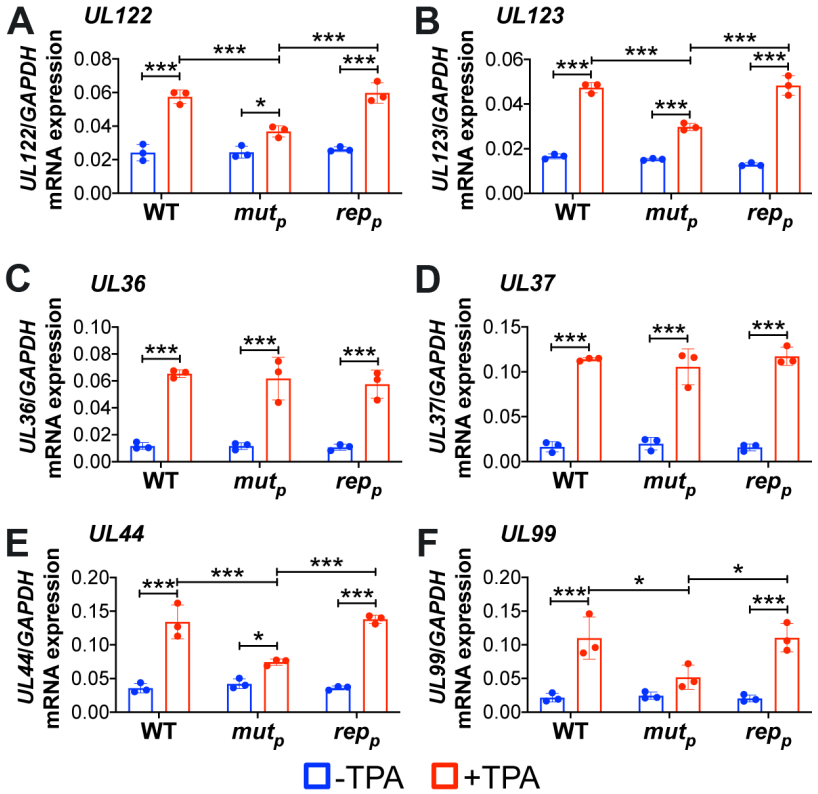

Figure 4. AP-1*mut$_p$* infection of Kasumi-3 cells impacts MIEP-driven gene expression, but not the transcription of other IE genes. Kasumi-3 cells were latently infected (moi = 1.0) with the indicated viruses. At 7 dpi, half of each infected population was cultured for an additional 2 d with vehicle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

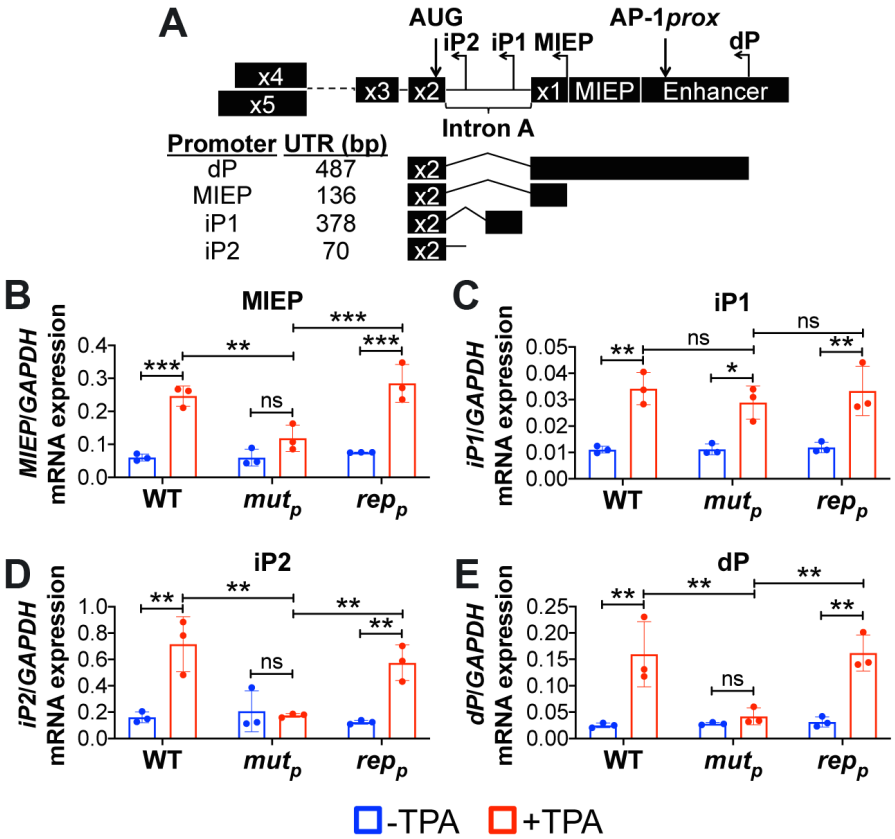

Figure 5. Transcripts driven from the canonical MIEP, iP2, and dP require AP-1 binding following reactivation. Kasumi-3 cells were infected (moi = 1.0) with the indicated viruses. At 7 dpi, half of each infected population was cultured for an additional 2 d with vehicle (DMSO; -TPA, blue bars) or TPA (+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,* plotted as arbitrary units. 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. Abbreviations: AP-1mut_p (mut_p), AP-1rep_p (rep_p).