

1 **CAGE-seq reveals that HIV-1 latent infection does not trigger unique cellular**
2 **responses in a Jurkat T cell model.**

3 Hiroyuki Matsui¹, Kotaro Shirakawa^{1*}, Yoshinobu Konishi^{1, 2}, Shigeki Hirabayashi^{1, 3},
4 Anamaria Daniela Sarca¹, Hirofumi Fukuda¹, Ryosuke Nomura¹, Emani Stanford¹,
5 Yoshihito Horisawa¹, Yasuhiro Kazuma¹, Tadahiko Matsumoto¹, Hiroyuki Yamazaki¹,
6 Yasuhiro Murakawa^{3, 4, 5, 6}, Emilie Battivelli⁷, Eric Verdin⁷, Yoshio Koyanagi⁸, Akifumi
7 Takaori-Kondo¹

8 From the ¹Department of Hematology and Oncology, Graduate School of Medicine, Kyoto
9 University, Kyoto, Japan, ²Department of Hematology, Kansai Electric Power Medical
10 Research Institute, Osaka, Japan, ³RIKEN Center for Integrative Medical Sciences,
11 Yokohama, Japan, ⁴Institute for the advanced study of human biology, Kyoto University,
12 Kyoto, Japan, ⁵Department of Medical Systems Genomics, Graduate School of Medicine,
13 Kyoto University, Kyoto, Japan. ⁶The FIRC Institute of Molecular Oncology (IFOM),
14 Milan, Italy, ⁷Buck Institute for Research on Aging, Novato, CA, USA, ⁸Laboratory of
15 Systems Virology, Institute for Frontier Life and Medical Sciences, Kyoto University,
16 Kyoto, Japan,

17 ***Correspondence:**

18 Kotaro Shirakawa, MD, PhD

19 Department of Hematology and Oncology, Graduate School of Medicine, Kyoto University

20 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan

21 Telephone: +81-75-751-4964, Fax: +81-75-751-4963

22 E-mail: kotash@kuhp.kyoto-u.ac.jp

23

24

25

26

27

28

29

30

31 **Abstract**

32 The cure for HIV-1 is currently stalled by our inability to specifically identify and
33 target latently infected cells. HIV-1 viral RNA/DNA or viral proteins are recognized by
34 cellular mechanisms and induce interferon responses in virus producing cells, but changes
35 in latently infected cells remain unknown. HIV_{GKO} contains a GFP reporter under the HIV-1
36 promoter and an mKO2 reporter under the internal EF1 α promoter. This viral construct
37 enables direct identification of HIV-1 both productively and latently infected cells. In this
38 study we aim to identify specific cellular transcriptional responses triggered by HIV-1 entry
39 and integration using Cap Analysis of Gene Expression (CAGE).

40 We deep sequenced CAGE tags in uninfected, latently and productively infected
41 cells and compared their differentially expressed transcription start site (TSS) profiles.
42 Virus producing cells had differentially expressed TSSs related to T-cell activation and
43 apoptosis when compared to uninfected cells or latently infected cells. Surprisingly, latently
44 infected cells had only 33 differentially expressed TSSs compared to uninfected cells.
45 Among these, SPP1 and APOE were down-regulated in latently infected cells. SPP1 or
46 APOE knockdown in Jurkat T cells increased susceptibility to HIV_{GKO} infection,

47 suggesting that they have anti-viral properties. Components of the PI3K/mTOR pathway,
48 MLST8, 4EBP and RPS6, were significant TSSs in productively infected cells, and S6K
49 phosphorylation was increased compared to latently infected cells, suggesting that mTOR
50 pathway activity plays a role in establishing the latent reservoir. These findings indicate that
51 HIV-1 entry and integration do not trigger unique transcriptional responses when infection
52 becomes latent.

53 **Importance**

54 Latent HIV-1 infection is established as early as the first viral exposure and
55 remains the most important barrier in obtaining the cure for HIV-1 infection. Here, we used
56 CAGE to compare the transcriptional landscape of latently infected cells with that of
57 non-infected or productively infected cells. We found that latently infected cells and
58 non-infected cells show quite similar transcriptional profiles. Our data suggest that T-cells
59 cannot recognize incoming viral components nor the integrated HIV-1 genome when
60 infection remains latent. These findings should guide future research into widening our
61 approaches to identify and target latent HIV-1 infected cells.

62

63 **Introduction**

64 HIV-1 infection has changed from a life-threatening disease to a chronic infectious
65 disease with the introduction of anti-retroviral therapy (ART). However, ART cannot
66 eradicate HIV-1, because HIV-1 persists in the human body in a latent state and starts to
67 replicate within weeks after cessation of ART. This latent infection occurs within long-lived
68 cells including resting CD4⁺ T cells, follicular dendritic cells, and hematopoietic stem cells
69 and can be established at a very early stage of infection (1). HIV-1 enters the cellular
70 nucleus and integrates into the human genome, targeting mainly the introns of actively
71 transcribed genes close to the nucleopore (2-4). HIV-1 transcription depends on the cellular
72 transcriptional machinery, therefore, the chromatin status of its integration site will greatly
73 affect the viral transcriptional outcome. If integration occurs in heterochromatin or within
74 genes that will later be inactivated, these integration events can lead to latent infection
75 directly after integration (5, 6). Current ART is capable of suppressing ongoing viral
76 transcription and new infections (7-9), therefore, to achieve an HIV cure, it is imperative to
77 elucidate the mechanism behind HIV-1 latent infection and the early events around HIV-1
78 integration.

79 Productive HIV-1 infection triggers cellular innate immune responses and affects
80 cellular membrane protein expression. For example, Interferon- γ Inducible Protein 16
81 (IFI16) and cyclic GMP-AMP synthase (cGAS) recognize HIV reverse transcription (RT)
82 products and activate the adaptor protein STING which then stimulates TBK1 and IRF3 to
83 induce the transcription of interferon genes (10, 11). HIV-1 evades this innate immune
84 response by interacting with host proteins such as Cyclophilin A (CypA) and
85 polyadenylation specificity factor subunit 6 (CPSF6) which protects RT products from
86 being detected by IFI16 and cGAS (12-14). Although a number of surface markers of
87 productive HIV-1 infection have been identified, including immune checkpoint molecules
88 (15), CD2 (16), CD30 (17) and CD32 (18), there are currently no established surface
89 markers that can distinguish latently infected cells from non-infected cells.

90 Various HIV latency models using primary CD4⁺ T-cells and reporter HIV
91 constructs have been developed for latency research (19). One of these constructs is
92 HIV_{GKO}, which contains two fluorescent reporters: codon-switched eGFP (csGFP) driven
93 by the HIV-1 LTR promoter and mKO2 driven by an internal elongation factor alpha
94 (EF1 α) promoter. HIV_{GKO} enables us to identify latently infected cells directly as mKO2

95 single positive cells and distinguish latently infected cells from non-infected and
96 productively infected cells (6, 20).

97 Single cell RNA-seq results of latently infected cells using primary cell models of
98 HIV-1 latency showed that latently infected cells express some memory T-cell markers
99 (*CCR7*, *SELL*, *CD27*), and reactivated cells express T-cell activation markers (*IL-2RA*,
100 *HLA-DR*, *CD38*) (21). Most transcriptome studies of HIV-1 latency compared the
101 transcriptome profile of latently infected cells to that of reactivated cells (22, 23). Thus,
102 there are no reports that directly compare the transcriptome profile of latently infected cells
103 to that of non-infected cells.

104 Cap Analysis of Gene Expression (CAGE) is a method for promoter identification
105 and transcription profiling by deep sequencing the 5' capped ends of transcripts that can
106 provide robust quantification of 5' ends of transcripts that exist at low levels (24, 25). In
107 this study, we use CAGE to perform a transcriptome analysis of a cell line model of HIV-1
108 latency. We found that the transcriptome profile of latently infected cells is quite similar to
109 that of non-infected cells and latent infection does not induce a specific cellular response.
110 Even though latently infected cells showed a very similar transcriptomic profiles to that of

111 uninfected cells, we found that SPP1 and APOE were significantly enriched in non-infected
112 compared to latently infected cells and knock down of these genes promoted HIV-1
113 infection. In contrast, we observed clear differences in the expression levels of three mTOR
114 signaling pathway genes, *MLST8*, *RPS6* and *EIF4EBP1*, between latently infected and
115 productively infected cells. We show that RPS6 knockdown increased phosphorylation of
116 S6K through negative feedback and resulted in an increase of productive infection.
117 Furthermore, S6K inhibitor treatment increased latent infection, suggesting that S6K
118 activity correlates with HIV-1 transcription and latency formation.

119 **Results**

120 **Characterization of the latency model using the dual-fluorescence HIV-1 reporter**

121 **HIV_{GKO}**

122 First, we infected Jurkat T cells with HIV_{GKO}, a dual reporter HIV-1 virus. The
123 schematic overview of our experiment is shown in Fig. 1A. Latently infected cells are
124 labeled as mKO single positive and productively infected cells are labeled as mKO and
125 csGFP double positive cells (Fig. 1B). Four days after infection, we FACS sorted the cells
126 into three fractions: non-infected (double negative), latently infected (mKO2 single

127 positive) and productively infected (csGFP and mKO2 double positive) cells and confirmed
128 that the purity of each cell fraction was more than 99 %.

129 Next, to confirm that the mKO single positive cells are truly latently infected cells,
130 we measured three HIV transcripts (Unspliced (US), Single spliced (SS) and Multiple
131 spliced (MS)) by RT-qPCR (6) and the relative copy number of integrated viral DNA by
132 Alu-PCR based quantitative PCR in each fraction. As expected, productively infected cells
133 showed high levels of all HIV transcripts while non-infected and latently infected cells
134 showed very low levels (Fig. 1C). In addition, the relative copy numbers of integrated
135 HIV_{GKO} in latently and productively infected cells were comparable (Fig. 1D). These
136 results confirmed that HIV_{GKO} was integrated into the genome but transcriptionally silent in
137 mKO2 single positive latently infected cells. We also detected HIV-1 integration 1.3% of
138 double negative cells using Alu-based quantitative genomic PCR (Fig. 1D), suggesting that
139 a small number of our non-infected cells contain HIV_{GKO} proviruses in which both parts of
140 the HIV-1 LTR and EF1 α promoters are deleted. Therefore, we designated the non-infected
141 cells as majority of cells (more than 98 %) are uninfected and a very few cells contain
142 defective HIV DNA.

143 We next evaluated the reactivation capacity of latently infected cells using three
144 latency-reversing agents (LRAs): TNF-alpha, PMA and vorinostat. Latently infected cells
145 were treated with these LRAs for 24h and the efficiency of reactivation was assessed by
146 flow cytometry. Whereas around 20 % of cells spontaneously reactivated in DMSO treated
147 cells, all three LRAs efficiently reactivated latently infected cells (Fig. 1E). This result
148 confirmed that these latently infected cells contain inducible proviruses.

149 **CAGE captures the transcription start site of HIV_{GKO} at single base resolution**

150 We performed CAGE for each FACS sorted HIV_{GKO} non-infected, latently infected,
151 productively infected and HIV_{GKO} unexposed Jurkat T cells. We obtained approximately 75
152 million CAGE tags from 75 thousand transcription start sites (TSSs) for each fraction (Fig.
153 2A). Among these, we detected 380 thousand HIV-1 derived CAGE tags which constituted
154 0.49 % of all detected CAGE tags. The genomic structure of HIV_{GKO} and detected CAGE
155 counts are shown in Fig. 2B. We identified the highest CAGE peak at the 5'-LTR in
156 productively infected cells and also identified high CAGE peaks at the EF1 α locus in both
157 latently infected and productively infected cells (Fig. 2C and D). This result also supports
158 that mKO single positive cells were HIV-1 transcriptionally silent. Notably, the highest

159 CAGE peak in the 5'-LTR was located at the U3/R boundary (Fig. 2B) which is consistent
160 with a previous study (26).

161 **HIV-1 latent infection does not trigger unique cellular responses**

162 We next compared CAGE tags from each of the sorted samples with unexposed
163 Jurkat cells and found that 965 TSSs were common among all three comparisons (Fig. 3A
164 and B). Furthermore, most of the highly significant TSSs in the comparison between
165 unexposed and latently infected Jurkat cells were common in all three comparisons (Fig.
166 3C and D). These results suggest that the differences between unexposed Jurkat cells and
167 latently infected Jurkat cells were not unique but rather common in non-infected cells and
168 productively infected cells. DAVID GO analysis (27, 28) showed that significantly enriched
169 biological processes between unexposed and latently infected cells were very similar to
170 those between unexposed and non-infected cells (Fig. 3E), suggesting that the infection and
171 consecutive responses of productively infected cells may have led to stimulation and
172 consecutive similar gene expression changes in both non-infected and latently infected cells
173 while the cells were cultured together. Therefore, we decided to focus on the differences
174 between non-infected, latently infected and productively infected cells.

175 To investigate the differences in gene expression profiles between these three
176 fractions, we performed hierarchical clustering. The gene expression profile of productively
177 infected cells was quite different from that of the other two fractions (Fig. 4A). Notably, we
178 found no major differences between non-infected and latently infected cells. Next, we
179 performed differential expression analysis to identify significant TSSs among non-infected,
180 latently infected and productively infected cells. Differential expression analysis identified
181 2,826 TSSs in the comparison between non-infected and productively infected cells and
182 1,816 TSSs in the comparison between latently infected and productively infected cells, but
183 only 33 TSSs were identified in the comparison between non-infected and latently infected
184 cells (Fig. 4B and Supplemental tables 1, 2 and 3). The number of significantly
185 differentially expressed TSSs between non-infected and latently infected cells was much
186 smaller than that between productively infected and non-infected or latently infected cells,
187 respectively. We further investigated the expression levels of individual genes that are
188 associated with IFN response. We did not observe any significant upregulation of IFN
189 genes (*IFN* α , *IFN* β and *IFN* γ) and genes that are associated with IFN response (*CGAS*,
190 *PPIA*, *TREX1*, *IRF3* and *TBK1*) in latently infected cells or productively infected cells
191 compared to non-infected cells; however, we detected a significant decrease of *IFI16* and a

192 significant increase of *STING* expression in productively infected cells (Fig. 5). Altogether,
193 these results suggest that HIV-1 productive infection affects genes involved in the pattern
194 recognition receptor (PRR) signaling pathway; however, HIV-1 latent infection does not
195 trigger any unique cellular transcriptional changes, including innate immune responses.

196 **Productive infection induces genes associated with apoptosis and T-cell activation**

197 Among the significant TSSs identified by differential expression analysis, 1,550
198 TSSs and 1,076 TSSs were up-regulated and 1,276 TSSs and 740 TSSs were
199 down-regulated in productively infected cells compared to non-infected and latently
200 infected cells respectively (Fig. 4B and Supplemental tables 1 and 2). We found that the
201 expression levels of T-cell activation markers (*CD69*, *CD70* and *CD38*) were significantly
202 increased in productively infected cells (Fig. 4C and D). Furthermore, we observed an
203 induction of pro-apoptotic genes (*GZMA*, *BAX* and *BCL2L11*) in productively infected cells
204 (Fig. 4C and D). Next, to comprehensively investigate which biological processes these
205 up-regulated TSSs in productively infected cells belong to, we performed DAVID GO
206 analysis. We confirmed that the up-regulated TSSs in productively infected cells belong to
207 T-cell signal transduction, immune response and apoptosis pathways (Fig. 4F). These

208 results suggest that T-cell activation is required for efficient virus production and that
209 productive infection induces pro-apoptotic genes resulting in cell death.

210 **APOE and SPP1 restrict HIV_{GKO} infection in both Jurkat T cells and primary CD4⁺ T**
211 **cells**

212 Differential expression analysis identified 33 significant TSSs between
213 non-infected and latently infected cells. Among these 33 TSSs, only 2 TSSs were
214 up-regulated in latently infected cells, but these TSSs were located within non-annotated
215 regions and did not express functional proteins (Fig. 4E and 6A and Supplemental table 3).
216 Therefore, we could not identify any specific biomarker for latently infected cells. On the
217 other hand, as illustrated in Fig. 6A, the expression levels of the other 31 TSSs were higher
218 in non-infected cells compared to both latently and productively infected cells. We assumed
219 that these up-regulated genes in non-infected cells might have putative anti-retroviral
220 activity for HIV-1. Among them, we focused on *APOE* and *SPP1* which both showed
221 significant enrichment in uninfected cells. Apolipoprotein E (APOE) is involved in
222 lipoprotein metabolism and is reported to inhibit HIV-1 infection in macrophages (29, 30).
223 SPP1, also known as osteopontin - a component of the extracellular matrix of bone,

224 suppresses T cell activation (31, 32) and promotes HIV-1 infection in macrophages (33). To
225 test if these proteins have anti-viral properties, we knocked down SPP1 and APOE in Jurkat
226 T cells and evaluated the changes in proportion of HIV_{GKO} infected cells by flow cytometry.
227 First, we established Jurkat T cell lines that express shRNA against SPP1, APOE or
228 luciferase (non-target control) in a doxycycline (DOX) inducible manner. We confirmed
229 that DOX treatment for 4 days knocked down SPP1 and APOE (Fig. 6B and D). Next, we
230 infected these cells lines with HIV_{GKO}, with or without prior DOX treatment, and analyzed
231 the proportion of infected cells by flow cytometry at 2 days post-infection. We found that
232 knock down of SPP1 and APOE significantly increased the percentage of infected cells (Fig.
233 6C and E).

234 To further confirm these findings in primary CD4⁺ T cells, we used siRNA to
235 knock down SPP1 and APOE in human primary CD4⁺ T cells and examined infectivity of
236 HIV_{GKO} by flow cytometry. We isolated primary CD4⁺ T cells from peripheral blood and
237 stimulated them with anti-CD3/CD28 beads for 3 days. We introduced siRNA against SPP1
238 and APOE into stimulated CD4⁺ T cells via electroporation and infected them with HIV_{GKO}
239 at 3 days post-transfection. We evaluated the proportion of HIV_{GKO} infected cells by flow

240 cytometry at 3 days post-infection. We confirmed the knock down of SPP1 and APOE with
241 RT-qPCR (Fig. 7A and B). As expected, knock down of SPP1 and APOE increased the
242 proportion of infected cells in human primary CD4⁺ T cells, respectively (Fig. 7C and D).
243 Taken together, these results support our hypothesis that SPP1 and APOE have an
244 anti-retroviral effect against HIV-1.

245 To investigate how SPP1 and APOE inhibit HIV infection, we evaluated the
246 amounts of late reverse transcription (RT) products, 2-LTR circles and integrated HIV upon
247 knock down of APOE and SPP1 in primary CD4⁺ T cells. Briefly, we introduced siRNA
248 and infected cells with HIV_{GKO} as described above, and measured the copy number of late
249 RT and 2-LTR products at 4, 8, 12- and 24-hours post-infection and of integrated HIV at 24
250 hours post-infection by Alu q-PCR. We detected an increase of late RT products (Fig. 7E
251 and F), relatively low levels of 2-LTR circles (Fig. 7G and H) and a significant increase of
252 integrated HIV upon either SPP1 or APOE knock down (Fig. 7I and J). These results
253 suggest that both SPP1 and APOE prevent HIV infection by interfering with integration and
254 possibly RT.

255

256 **Increased phosphorylation of S6K helps prevent latency**

257 The mammalian target of rapamycin (mTOR) signaling pathway is associated with
258 the regulation of cell proliferation as well as lipid and amino acid metabolism (34).
259 Genome-wide functional screening using a shRNA library revealed that the suppression of
260 the mTOR complex prevents CD3/CD28 mediated reactivation of latently infected cells
261 and mTOR inhibitors suppress CD3/CD28 mediated reactivation of latently infected cells
262 (35). In addition, genetic depletion of TSC1, that inhibits the activation of the mTORC1
263 complex through the phosphorylation of Rheb, promotes the reactivation of latently
264 infected cells (36). These results suggest that the mTOR signaling pathway plays a role in
265 reactivating latently infected cells, but it remains unknown whether mTOR also plays a role
266 in establishing the latent reservoir. We investigated the expression levels of genes that
267 compose the mTOR signaling pathway and found that there were significant differences in
268 the expression levels of *MLST8*, *EIF4EBP1* and *RPS6* between latently infected and
269 productively infected cells (Fig. 8A, B and C, respectively), suggesting that the activity of
270 the mTOR signaling pathway has some influence on establishing latency. To confirm this
271 possibility, we established cell lines that stably express shRNA against *MLST8*, *4EBP1* or

272 RPS6 in a doxycycline inducible manner and confirmed efficient knock down of each
273 protein after 4 days of DOX treatment (Fig. 8D, E and F, respectively). We then infected
274 these cells with HIV_{GKO} and evaluated the proportion of infected cells by flow cytometry.
275 We observed that knock down of RPS6 decreased the percentage of latently infected cells
276 and increased that of productively infected cells while the overall infection (latent +
277 productive infections) rate was largely the same. However, the knock down of MLST8 and
278 4EBP1 had no effect on infection rates or type (Fig. 8G, H and I). Based on these results,
279 we hypothesized that knock down of RPS6 would increase the phosphorylation of upstream
280 components of the mTOR signaling pathway and examined the phosphorylation state of
281 mTOR and S6K. As expected, knock down of RPS6 increased the expression and
282 phosphorylation of S6K (Fig. 8J). We observed no differences in the expression or
283 phosphorylation of mTOR upon knock down of RPS6 (Fig. 8J). Furthermore, we found that
284 productively infected cells had higher levels of phosphorylated S6K compared to
285 non-infected and latently infected cells (Fig. 8K). Expression of S6K itself was also
286 increased, possibly because phosphorylation of S6K at T389 stabilizes S6K itself by
287 preventing proteasome degradation induced by JNK1-mediated phosphorylation of S6K at
288 S424 (37). S6K phosphorylates CDK9, a component of the p-TEFb complex, and plays an

289 important role in the reactivation of latently infected cells (35). We used selective S6K
290 inhibitors (LY2584702 and PF-4708671) to investigate whether S6K has an impact on
291 establishing the latent reservoir. Both S6K inhibitors increased the size of the latent
292 reservoir (Fig. 8L and M). The increase of the HIV latently infected cells by S6K inhibition
293 was also confirmed in primary CD4⁺ T cells (Fig. 9). Taken together, these results suggest
294 that HIV infection tends to become productive when the phosphorylation of S6K in
295 infected cells is relatively high, while it tends to become latent when it is relatively low
296 level.

297

298 Discussion

299 In this study, we demonstrate that latent HIV infection does not trigger a unique
300 cellular response in T-cells, while, on the other hand, productive infection enhances T-cell
301 activation and induces pro-apoptotic responses. In addition, we show that among the genes
302 that are up-regulated in non-infected cells compared to latently infected cells, APOE and
303 SPP1 have anti-retroviral properties in T-cells. Finally, we reveal that phosphorylation of
304 S6K is increased in productively infected cells and inhibiting S6K suppresses productive

305 infection and promotes latent infection.

306 A prior study using HIV infected cells derived from untreated viremic patients
307 showed that infected cells express ICOS, a critical co-stimulator of follicular helper T-cells
308 indicating recent cell activation (38). Bradley et al. also reported T-cell activation genes
309 were up-regulated in productively infected cells (21). In line with these findings, we
310 observed the up-regulation of some T-cell activation markers in productively infected cells.
311 In addition, it is widely accepted that productive infection results in apoptotic cell death
312 through a viral cytopathic effect and thought to be a mechanism that kills reactivated cells
313 (39-41). Consistent with these reports, we found that HIV infection causes up-regulation of
314 pro-apoptotic genes in productively infected cells, but not in latently infected cells. A recent
315 publication demonstrated that the anti-apoptotic protein BIRC5 and its upstream regulator
316 OX40 were up-regulated in both productively and latently infected CD4⁺ T-cells and can
317 promote survival of HIV-1 infected CD4⁺ T-cells (42). In contrast, we observed the
318 down-regulation of OX40 in productively infected cells (data not shown), but its expression
319 was maintained in latently infected cells. Overall, our results together with these various
320 observations suggest that productive infection leads to an apoptotic response in infected

321 cells, but latent infection allows cells to escape from such a response and survive to form
322 the latent reservoir.

323 Two pattern recognition receptors (PRRs), IFI16 and cGAS, play central roles in
324 sensing HIV-1 and inducing subsequent interferon responses (10, 11). These PRRs
325 recognize HIV-1 reverse transcription (RT) products, then activate STING and induce
326 interferon responses (10, 11). On the other hand, HIV-1 capsid prevents host innate immune
327 responses by interacting with CypA and CPSF6, allowing RT products to escape from being
328 recognized by these PRRs (12-14). The HIV-1 accessory protein Vpu also suppresses host
329 innate immune responses by degrading interferon regulatory factor 3 (IRF3), a central
330 transcription factor that drives host cell innate immunity (43). Moreover, cytosolic
331 exonuclease TREX1 degrades excess RT products and supports HIV-1 to evade innate
332 immune recognition and successfully infect the host cell (44). Therefore, it remains
333 unknown whether HIV latent infection triggers innate immune responses. In this study, we
334 did not observe innate immune responses in latently infected or productively infected cells.
335 This could be in part because we used a vesicular stomatitis virus G (VSV-G) pseudotyped
336 replication incompetent single round infection system while Vermeire et al. used a

337 spreading infection system to report that HIV-1 infection induces interferon responses in
338 primary CD4⁺ T cells (45). As a result, we may have underestimated the innate immune
339 response against HIV-1. Overall, these results provide new insights into the host innate
340 immune response against latent HIV infection.

341 SPP1 is known as a chemoattractant that promotes the migration of macrophages
342 and it is also known as a pro-inflammatory cytokine that enhances Th1 cytokine production
343 (46). The plasma and cerebrospinal fluid levels of SPP1 are well correlated with the
344 neuropsychological status of HIV-1 infected patients (47). We found that *SPP1* was
345 up-regulated in non-infected cells, suggesting that the presence of SPP1 induces distant
346 anti-viral responses that protect cells from further infection. We also found that knock down
347 of SPP1 increased the susceptibility of T-cells to HIV-1 infection. In contrast, a previous
348 study showed that SPP1 promotes HIV-1 infection in human derived macrophages through
349 the downregulation of I κ B α and subsequent activation of NF- κ B (33). Our results suggest
350 that SPP1 prevents HIV infection by interrupting RT or integration and plays a different
351 role in T cells than in macrophages. In addition, SPP1 is a cytokine and ligand for integrin
352 α v β 5 and CD44 and further loss of SPP1 increases the activity of CD4 and CD8 T cells (31,

353 32). Therefore, SPP1 may indirectly prevent HIV-1 infection through these cytokine signals.

354 Altogether, our findings shed light on the role of SPP1 in HIV infection but further

355 investigation is required to fully elucidate the role of SPP1 in HIV-1 infection.

356 Apolipoprotein E (APOE) is a small secreted protein involved in cellular lipid

357 metabolism and cholesterol transport. The relationship between *APOE* polymorphisms and

358 the development of Alzheimer's disease is well studied and the *APOE* E4 allele is a risk

359 factor for Alzheimer's disease. The relationship between *APOE* polymorphisms and

360 HIV-associated neurocognitive disease (HAND) was also extensively studied but the results

361 are still controversial (48-53). Regarding the relationship between APOE and HIV

362 infectivity, one report showed that the APOE E4 isoform protein enhances HIV-1 infection

363 (54). In general, however, APOE is known as a restriction factor of HIV and various studies

364 have proposed various mechanisms of how APOE inhibits HIV infection (29, 30, 55, 56).

365 Among these, two studies showed that APOE disrupted HIV infection at the HIV entry or

366 RT steps. In the present study, we found that the expression of APOE was increased in

367 non-infected cells compared to latently infected and productively infected cells and knock

368 down of APOE promoted HIV-1 infection in T-cells by interfering with viral RT or

369 integration. Thus, our findings are consistent with previous studies that support the notion
370 that APOE is an HIV-1 restriction factor.

371 The mTOR signaling pathway plays a central role in coordinating cell growth and
372 metabolism with extracellular stimuli (34, 57). mTOR inhibitors perturb HIV-1 infection by
373 inhibiting various steps of the HIV-1 life cycle (58, 59). Thus, the mTOR pathway plays a
374 role in efficient HIV-1 infection. Recently, Besnard et al. identified the mTOR complex as a
375 modulator of HIV latency by conducting genome-wide screening with a pooled
376 ultracomplex shRNA library and revealed that mTOR inhibitors suppressed reactivation of
377 latently infected CD4⁺ T cells (35). Moreover, several studies showed that suppression of
378 the mTOR signaling pathway prevents reactivation of latently infected cells (36, 60).
379 Altogether, it seems that the mTOR signaling pathway supports efficient virus production
380 and inhibition of mTOR promotes HIV latency. However, it remains unclear whether low
381 activity of the mTOR signaling pathway influences latency establishment. We demonstrated
382 that knock down of RPS6 up-regulated the mTOR signaling pathway through increased
383 phosphorylation of S6K and decreased the proportion of latently infected cells. We further
384 identified that S6K phosphorylation increased in productively infected cells and that S6K

385 inhibitors suppress productive infection and promote latent infection. S6K is known to
386 phosphorylate CDK9, which is a component of the p-TEFb complex that serves as a
387 cofactor of Tat-mediated transcription (35, 61). These findings suggest that the activity of
388 the mTOR signaling pathway, especially the phosphorylation level of S6K, in infected cells
389 has a significant influence not only on reversing latency but also on establishing latency
390 and suppression of S6K promotes infected cells to become latent. Taken together, these data
391 provide a new mechanistic insight into how the HIV-1 reservoir is established.

392 In conclusion, we used an *in vitro* latency model that can directly and
393 prospectively differentiate latently infected cells from non-infected and productively
394 infected cells to perform a transcriptome analysis of each of these cellular fractions. We
395 used the CAGE method to perform this analysis, which can precisely analyze the
396 transcriptome at individual transcription start site levels and demonstrated that HIV-1
397 productive infection enhances T-cell activation and induces pro-apoptotic responses, while
398 the integrated HIV itself is no longer recognized by host cellular immune systems when the
399 infection becomes latent. We found that *APOE* and *SPP1* are up-regulated in non-infected
400 cells and have anti-viral properties in T-cells. In addition, we also revealed that the

401 increased phosphorylation of S6K promotes productive infection and thereby inhibition of
402 S6K promotes latent infection. Overall, our study provides a new insight into the cellular
403 response induced by HIV-1 latent infection and the molecular mechanism that is associated
404 with establishing the latent HIV-1 reservoir.

405

406 **Materials and Methods**

407 **Cell lines and Plasmids**

408 Jurkat T cells were cultured in RPMI supplemented with 10% fetal bovine serum
409 (FBS) and 1% penicillin/streptomycin/glutamine (PSG). Primary CD4⁺ T cells were
410 cultured in RPMI supplemented with 10 % FBS, 1% PSG and 100 IU/ml human
411 recombinant IL-2 (SHIONOGI Co.). HIV_{GKO} was described in a previous study (6).
412 Tet-pLKO-puro was purchased from Addgene (Plasmid #21915). We generated
413 Tet-pLKO-puro vectors that express shRNA against luciferase (non-target control), SPP1,
414 APOE, MLST8, 4EBP1, RPS6 #1 and RPS6 #2 by inserting synthesized double-stranded
415 oligonucleotides between the EcoRI and AgeI restriction sites. Sequences of the
416 synthesized oligonucleotides are provided in Supplemental table 4.

417 **Virus Production**

418 HIV_{GKO} viral stocks were generated by co-transfecting Lenti-X 293T cells with a
419 plasmid encoding HIV_{GKO} and a plasmid encoding VSV-g envelope. Supernatants were
420 collected 48h after transfection, centrifuged (2,000 rpm, 20 min, RT), filtered through 0.45
421 micrometer pore-size PVDF membranes and concentrated by ultracentrifugation (25,000
422 rpm, 2h, 4 °C). Concentrated viral particles were resuspended in RPMI medium and stored
423 at -80 °C. Virus concentration was measured using the HIV-1 p24 Antigen ELISA kit
424 (ZeptoMetrix) according to the manufacturer's protocol. For the lentivirus expressing
425 shRNA against luciferase, SPP1, APOE, MLST8, 4EBP1 and RPS6, we co-transfected
426 Lenti-X 293T cells with each Tet-pLKO-puro plasmid described above and the
427 Trans-Lentiviral packaging plasmid mix (GE Dharmacon). Supernatants were collected,
428 concentrated and stocked as described above.

429 **HIV_{GKO} infection and sorting**

430 Jurkat T cells were infected with VSV-g pseudotyped HIV_{GKO} at a concentration of
431 15 ng of p24 per 3 x 10⁵ cells in 24 well plates. Infected cells were washed twice with PBS
432 at 24h after infection and analyzed or sorted at 4 or 5 days after infection by FACS Aria II.

433 Primary CD4⁺ T cells were spinoculated with VSV-g pseudotyped HIV_{GKO} at a
434 concentration of 70 ng of p24 per 1.5 x 10⁵ cells for 2 hours at 900 g at 32 °C. Infected cells
435 were cultured in the presence of 100 IU/ml IL-2 and analyzed at 3 days post infection by
436 FACS Aria II.

437 **DNA extraction and quantitative PCR**

438 Genomic DNA was extracted from cells using the QuickGene DNA whole blood
439 kit S (KURABO) according to the manufacturer's protocol. Time course determinations of
440 late RT products and quantification of 2-LTR circles were performed as described
441 previously (62). Integrated HIV_{GKO} genome was selectively amplified from the isolated
442 genomic DNA by using a forward primer located in the Alu sequence and a reverse primer
443 located in the Gag sequence of HIV_{GKO}. In the 2nd PCR step, the amplified integrated
444 HIV_{GKO} genome was measured by quantitative PCR using primers located in the 5'-LTR of
445 HIV_{GKO}. The primer sequences are provided in Supplemental table 4.

446 **RNA extraction and RT-qPCR**

447 Total RNA was extracted using the High Pure RNA Isolation kit (Roche). cDNA
448 was synthesized using ReverTra Ace qPCR RT Master Mix with gDNA remover

449 (TOYOBO LIFE SCIENCE) with a combination of random and oligo-dT primers.

450 Real-Time PCR was performed using TB Green Premix ExTaq II (TaKaRa). qPCR levels

451 of cell-associated HIV mRNA copy number and expression of APOE and SPP1 were

452 normalized to endogenous GAPDH expression. The primer sequences are provided in

453 Supplemental table 4.

454 **Protein extraction and Western blotting**

455 Cells were lysed with SDS sample buffer (62.5 mM Tris-HCl, 2 % SDS, 2 %

456 Glycerol). Protein content was determined using the Pierce BCA Protein assay kit

457 (ThermoFisher). Whole cell lysates were subject to immunoblot analysis using anti-MLST8

458 (GβL) (CST), anti-phospho-4EBP1(T37/46) (CST), anti-4EBP1 (CST), anti-phospho RPS6

459 (S240/244) (Merck), anti-RPS6 (Abcam), anti-phospho-p70-S6K (T389) (CST),

460 anti-p70-S6K (CST), anti-phospho mTOR (S2448) (CST), anti-mTOR (CST) and

461 anti-tubulin (Sigma) antibodies.

462 **CAGE library preparation**

463 Total RNA from each sample was extracted with miRNeasy Mini Kit (QIAGEN).

464 CAGE libraries were constructed using total RNAs based on the no-amplification

465 non-tagging CAGE libraries for Illumina next-generation sequencers (nAnT-iCAGE)
466 protocol (63). Briefly, first strand cDNAs were synthesized through reverse transcription
467 using random primers. Subsequently, 5' caps of RNAs were oxidized and biotinylated.
468 Cells were then treated with RNase I to remove single-stranded RNAs. Biotinylated
469 RNA/cDNA molecules were purified using streptavidin magnetic beads. Single-stranded
470 cDNAs were obtained by treating with RNase H and RNase I. After ligation of adapters and
471 second strand synthesis of cDNAs, CAGE libraries were finalized. CAGE libraries were
472 sequenced with NextSeq 500 (Illumina) in single-read mode.

473 **CAGE data analysis**

474 HIV_{GKO} sequence and human reference genome hg19 were combined, and the
475 index for the combined sequence was generated using HISAT2 version 2.0.5 (64) with the
476 default parameters. Raw reads were trimmed and aligned to the combined sequence using
477 HISAT2 with the following parameters: hisat2 -p 12 --trim3 2 -S. Next, generated SAM
478 files were converted into BAM files, and uniquely mapped reads with high mapping quality
479 values ($\text{MAPQ} \geq 20$) were retained using samtools version 1.9 (65). Using bedtools v2.25.0
480 (Quinlan, Bioinformatics 2010) with the parameters of genomecov -5 -bg -strand + or

481 genomecov -5 -bg -strand -, coverage of 5' ends of reads were computed on
482 FANTOM5-defined promoters (25) that were downloaded from
483 http://fantom.gsc.riken.jp/5/datafiles/latest/extra/CAGE_peaks/. The resultant bedGraph
484 files were converted to bigWig files with bedGraphToBigWig (66). Reads mapped to
485 promoters were counted using bigWigAverageOverBed (66). Promoters with no expression
486 in any sample were filtered out. After normalization by relative log expression, differential
487 expression analysis was carried out with edgeR version 3.16.5 (67), and q values were
488 calculated using the Benjamini–Hochberg method (68). For alignment to the HIV_{GKO}
489 sequence, each read was allowed to multimap to at most 2 loci because 5' LTR has the same
490 sequence arrangement as 3' LTR. Multimapping reads were divided equally among
491 candidates. CAGE signals in the HIV_{GKO} sequence were visualized using Integrative
492 Genomics Viewer (69).

493 Enrichment analysis was conducted on the identified significantly differentially
494 expressed genes ($p < 0.05$) using DAVID Functional Annotation Tool (27, 28). The
495 resulting p values were corrected with the Benjamini-Hochberg method (68).

496 **Lentiviral shRNA transduction**

497 To establish Jurkat T cells that were stably transduced with inducible shRNA
498 against luciferase, APOE, SPP1, MLST8, 4EBP1, RPS6 #1 and RPS6 #2, Jurkat T cells
499 were infected with lentivirus containing inducible shRNA against each gene and treated
500 with puromycin 48h post-transduction to select stably transduced cells. The selected cells
501 were maintained in RPMI supplemented with 10 % FBS, 1 % PSG and 1 µg/ml of
502 puromycin.

503 **Primary CD4⁺ T cell isolation and activation**

504 Primary CD4⁺ T cells were isolated from human peripheral blood using
505 RosetteSep Human CD4⁺ T cell Enrichment Cocktail (StemCell Technologies) according to
506 the manufacturer's protocol. Isolated primary CD4⁺ T cells were stimulated with
507 CD3/CD28 activation beads (Gibco) at a concentration of 0.5 bead /cell in the presence of
508 100 IU/ml IL-2 for 3 days.

509 **siRNA electroporation**

510 siRNAs were purchased from Thermo Fisher SCIENTIFIC (SPP1; Silencer™
511 select pre-Designed siRNA s13375, s13376, APOE; Silencer™ select pre-Designed siRNA
512 s1495, s194291 non-target control; Silencer™ Select Negative Control

513 #1 siRNA (4390843)). Purified CD4⁺ T cells were stimulated with CD3/CD28 activation
514 beads for 3 days prior to electroporation. siRNA against each gene or non-target control
515 was then introduced into activated CD4⁺ T cells via electroporation at a concentration of
516 300 nM per 1.0 x 10⁶ cells. Electroporation was performed using the T-023 program on the
517 Nucleofector 2b (Lonza). After electroporation, cells were returned to culture and were kept
518 stimulated with CD3/CD28 activation beads until HIV_{GKO} infection.

519 **Statistical Analysis**

520 Significance in quantitative PCR and flow cytometry was analyzed by Student's
521 t-test, and marked with * in the manuscript.

522 **Data Availability**

523 Raw and processed data are available from Gene Expression Omnibus under accession
524 GSE149492.

525

526 **Acknowledgements**

527 This work was partly supported by JSPS KAKENHI Grant Numbers 19H03502, 18H03992
528 to A.T-K., 16K08809, 19K07591 to K.S., 18H03992 to Y.M., and by AMED under Grant
529 Numbers 20fk0410011, 20fk0410034, 20fk0410014 to A. T-K., 20fk0410014 to Y.K., Joint
530 Usage/Research Center program of Institute for Frontier Life and Medical Sciences, Kyoto
531 University to A.T-K.

532

533 **Competing Interests:** The authors declare no competing interests.

534

535 **Author contributions**

536 H.M., K.S., and A.T.-K. conceived and designed the study.; H.M. and Y.K. performed
537 experiments and data analysis with help from S.H., A.D.S., H.F., R.N., E.S., Y.H., Y.K.,
538 T.M., H.Y., Y.M. and Y.K.; S.H., Y.M. performed CAGE library preparation and data
539 processing; E.B. and E.V. provided vital reagents.; H.M., Y.K., S.H., A.D.S., K.S., E.V. and
540 A.T.-K. wrote the paper. All the authors reviewed and approved the manuscript.

541

542 **References**

- 543 1. Chun TW, Engel D, Berrey MM, Shea T, Corey L, Fauci AS. 1998. Early establishment of a pool of
544 latently infected, resting CD4(+) T cells during primary HIV-1 infection. *Proc Natl Acad Sci U S A*
545 95:8869-73.
- 546 2. Ciuffi A, Llano M, Poeschla E, Hoffmann C, Leipzig J, Shinn P, Ecker JR, Bushman F. 2005. A role
547 for LEDGF/p75 in targeting HIV DNA integration. *Nat Med* 11:1287-9.
- 548 3. Meehan AM, Saenz DT, Morrison JH, Garcia-Rivera JA, Peretz M, Llano M, Poeschla EM. 2009.
549 LEDGF/p75 proteins with alternative chromatin tethers are functional HIV-1 cofactors. *PLoS Pathog*
550 5:e1000522.
- 551 4. Schrijvers R, De Rijck J, Demeulemeester J, Adachi N, Vets S, Ronen K, Christ F, Bushman FD,
552 Debyser Z, Gijsbers R. 2012. LEDGF/p75-independent HIV-1 replication demonstrates a role for
553 HRP-2 and remains sensitive to inhibition by LEDGIns. *PLoS Pathog* 8:e1002558.
- 554 5. Chavez L, Calvanese V, Verdin E. 2015. HIV Latency Is Established Directly and Early in Both
555 Resting and Activated Primary CD4 T Cells. *PLoS Pathog* 11:e1004955.
- 556 6. Battivelli E, Dahabieh MS, Abdel-Mohsen M, Svensson JP, Tojal Da Silva I, Cohn LB, Gramatica A,
557 Deeks S, Greene WC, Pillai SK, Verdin E. 2018. Distinct chromatin functional states correlate with
558 HIV latency reactivation in infected primary CD4(+) T cells. *Elife* 7.
- 559 7. Josefsson L, von Stockenstrom S, Faria NR, Sinclair E, Bacchetti P, Killian M, Epling L, Tan A, Ho
560 T, Lemey P, Shao W, Hunt PW, Somsouk M, Wylie W, Douek DC, Loeb L, Custer J, Hoh R, Poole L,
561 Deeks SG, Hecht F, Palmer S. 2013. The HIV-1 reservoir in eight patients on long-term suppressive
562 antiretroviral therapy is stable with few genetic changes over time. *Proc Natl Acad Sci U S A*
563 110:E4987-96.
- 564 8. Kearney MF, Spindler J, Shao W, Yu S, Anderson EM, O'Shea A, Rehm C, Poethke C, Kovacs N,
565 Mellors JW, Coffin JM, Maldarelli F. 2014. Lack of detectable HIV-1 molecular evolution during
566 suppressive antiretroviral therapy. *PLoS Pathog* 10:e1004010.
- 567 9. McManus WR, Bale MJ, Spindler J, Wiegand A, Musick A, Patro SC, Sobolewski MD, Musick VK,
568 Anderson EM, Cyktor JC, Halvas EK, Shao W, Wells D, Wu X, Keele BF, Milush JM, Hoh R,
569 Mellors JW, Hughes SH, Deeks SG, Coffin JM, Kearney MF. 2019. HIV-1 in lymph nodes is
570 maintained by cellular proliferation during antiretroviral therapy. *J Clin Invest* 130:4629-4642.
- 571 10. Jakobsen MR, Bak RO, Andersen A, Berg RK, Jensen SB, Tengchuan J, Laustsen A, Hansen K,
572 Ostergaard L, Fitzgerald KA, Xiao TS, Mikkelsen JG, Mogensen TH, Paludan SR. 2013. IFI16
573 senses DNA forms of the lentiviral replication cycle and controls HIV-1 replication. *Proc Natl Acad*
574 *Sci U S A* 110:E4571-80.
- 575 11. Gao D, Wu J, Wu YT, Du F, Aroh C, Yan N, Sun L, Chen ZJ. 2013. Cyclic GMP-AMP synthase is an

- innate immune sensor of HIV and other retroviruses. *Science* 341:903-6.
12. Hatzioannou T, Perez-Caballero D, Cowan S, Bieniasz PD. 2005. Cyclophilin interactions with incoming human immunodeficiency virus type 1 capsids with opposing effects on infectivity in human cells. *J Virol* 79:176-83.
13. Li XD, Wu J, Gao D, Wang H, Sun L, Chen ZJ. 2013. Pivotal roles of cGAS-cGAMP signaling in antiviral defense and immune adjuvant effects. *Science* 341:1390-4.
14. Rasaiyaah J, Tan CP, Fletcher AJ, Price AJ, Blondeau C, Hilditch L, Jacques DA, Selwood DL, James LC, Noursadeghi M, Towers GJ. 2013. HIV-1 evades innate immune recognition through specific cofactor recruitment. *Nature* 503:402-405.
15. Fromentin R, Bakeman W, Lawani MB, Khoury G, Hartogensis W, DaFonseca S, Killian M, Epling L, Hoh R, Sinclair E, Hecht FM, Bacchetti P, Deeks SG, Lewin SR, Sekaly RP, Chomont N. 2016. CD4+ T Cells Expressing PD-1, TIGIT and LAG-3 Contribute to HIV Persistence during ART. *PLoS Pathog* 12:e1005761.
16. Iglesias-Ussel M, Vandergeeten C, Marchionni L, Chomont N, Romerio F. 2013. High levels of CD2 expression identify HIV-1 latently infected resting memory CD4+ T cells in virally suppressed subjects. *J Virol* 87:9148-58.
17. Hogan LE, Vasquez J, Hobbs KS, Hanhauser E, Aguilar-Rodriguez B, Hussien R, Thanh C, Gibson EA, Carvidi AB, Smith LCB, Khan S, Trapecar M, Sanjabi S, Somsouk M, Stoddart CA, Kuritzkes DR, Deeks SG, Henrich TJ. 2018. Increased HIV-1 transcriptional activity and infectious burden in peripheral blood and gut-associated CD4+ T cells expressing CD30. *PLoS Pathog* 14:e1006856.
18. Descours B, Petitjean G, Lopez-Zaragoza JL, Bruel T, Raffel R, Psomas C, Reynes J, Lacabartz C, Levy Y, Schwartz O, Lelievre JD, Benkirane M. 2017. CD32a is a marker of a CD4 T-cell HIV reservoir harbouring replication-competent proviruses. *Nature* 543:564-567.
19. Spina CA, Anderson J, Archin NM, Bosque A, Chan J, Famiglietti M, Greene WC, Kashuba A, Lewin SR, Margolis DM, Mau M, Ruelas D, Saleh S, Shirakawa K, Siliciano RF, Singhania A, Soto PC, Terry VH, Verdin E, Woelk C, Wooden S, Xing S, Planelles V. 2013. An in-depth comparison of latent HIV-1 reactivation in multiple cell model systems and resting CD4+ T cells from aviremic patients. *PLoS Pathog* 9:e1003834.
20. Calvanese V, Chavez L, Laurent T, Ding S, Verdin E. 2013. Dual-color HIV reporters trace a population of latently infected cells and enable their purification. *Virology* 446:283-92.
21. Bradley T, Ferrari G, Haynes BF, Margolis DM, Browne EP. 2018. Single-Cell Analysis of Quiescent HIV Infection Reveals Host Transcriptional Profiles that Regulate Proviral Latency. *Cell Rep* 25:107-117 e3.

- 609 22. Golumbeanu M, Cristinelli S, Rato S, Munoz M, Cavassini M, Beerenwinkel N, Ciuffi A. 2018.
 610 Single-Cell RNA-Seq Reveals Transcriptional Heterogeneity in Latent and Reactivated HIV-Infected
 611 Cells. *Cell Rep* 23:942-950.
- 612 23. Cohn LB, da Silva IT, Valieris R, Huang AS, Lorenzi JCC, Cohen YZ, Pai JA, Butler AL, Caskey M,
 613 Jankovic M, Nussenzweig MC. 2018. Clonal CD4(+) T cells in the HIV-1 latent reservoir display a
 614 distinct gene profile upon reactivation. *Nat Med* 24:604-609.
- 615 24. Murakawa Y, Yoshihara M, Kawaji H, Nishikawa M, Zayed H, Suzuki H, Fantom C, Hayashizaki Y.
 616 2016. Enhanced Identification of Transcriptional Enhancers Provides Mechanistic Insights into
 617 Diseases. *Trends Genet* 32:76-88.
- 618 25. Consortium F, the RP, Clst, Forrest AR, Kawaji H, Rehli M, Baillie JK, de Hoon MJ, Haberle V,
 619 Lassmann T, Kulakovskiy IV, Lizio M, Itoh M, Andersson R, Mungall CJ, Meehan TF, Schmeier S,
 620 Bertin N, Jorgensen M, Dimont E, Arner E, Schmidl C, Schaefer U, Medvedeva YA, Plessy C,
 621 Vitezic M, Severin J, Sempé C, Ishizu Y, Young RS, Francescato M, Alam I, Albanese D, Altschuler
 622 GM, Arakawa T, Archer JA, Arner P, Babina M, Rennie S, Balwierz PJ, Beckhouse AG,
 623 Pradhan-Bhatt S, Blake JA, Blumenthal A, Bodega B, Bonetti A, Briggs J, Brombacher F, Burroughs
 624 AM, Califano A, et al. 2014. A promoter-level mammalian expression atlas. *Nature* 507:462-70.
- 625 26. Kharytonchyk S, Monti S, Smaldino PJ, Van V, Bolden NC, Brown JD, Russo E, Swanson C, Shuey
 626 A, Telesnitsky A, Summers MF. 2016. Transcriptional start site heterogeneity modulates the structure
 627 and function of the HIV-1 genome. *Proc Natl Acad Sci U S A* 113:13378-13383.
- 628 27. Huang da W, Sherman BT, Lempicki RA. 2009. Bioinformatics enrichment tools: paths toward the
 629 comprehensive functional analysis of large gene lists. *Nucleic Acids Res* 37:1-13.
- 630 28. Huang da W, Sherman BT, Lempicki RA. 2009. Systematic and integrative analysis of large gene
 631 lists using DAVID bioinformatics resources. *Nat Protoc* 4:44-57.
- 632 29. Siddiqui R, Suzu S, Ueno M, Nasser H, Koba R, Bhuyan F, Noyori O, Hamidi S, Sheng G,
 633 Yasuda-Inoue M, Hishiki T, Sukegawa S, Miyagi E, Strebel K, Matsushita S, Shimotohno K, Ariumi
 634 Y. 2018. Apolipoprotein E is an HIV-1-inducible inhibitor of viral production and infectivity in
 635 macrophages. *PLoS Pathog* 14:e1007372.
- 636 30. Khan N, Datta G, Geiger JD, Chen X. 2018. Apolipoprotein E isoform dependently affects
 637 Tat-mediated HIV-1 LTR transactivation. *J Neuroinflammation* 15:91.
- 638 31. Klement JD, Paschall AV, Redd PS, Ibrahim ML, Lu C, Yang D, Celis E, Abrams SI, Ozato K, Liu K.
 639 2018. An osteopontin/CD44 immune checkpoint controls CD8+ T cell activation and tumor immune
 640 evasion. *J Clin Invest* 128:5549-5560.
- 641 32. Wei J, Marisetty A, Schrand B, Gabrusiewicz K, Hashimoto Y, Ott M, Grami Z, Kong LY, Ling X,

- 642 Caruso H, Zhou S, Wang YA, Fuller GN, Huse J, Gilboa E, Kang N, Huang X, Verhaak R, Li S,
 643 Heimberger AB. 2019. Osteopontin mediates glioblastoma-associated macrophage infiltration and is
 644 a potential therapeutic target. *J Clin Invest* 129:137-149.
- 645 33. Brown A, Islam T, Adams R, Nerle S, Kamara M, Eger C, Marder K, Cohen B, Schifitto G,
 646 McArthur JC, Sacktor N, Pardo CA. 2011. Osteopontin enhances HIV replication and is increased in
 647 the brain and cerebrospinal fluid of HIV-infected individuals. *J Neurovirol* 17:382-92.
- 648 34. Saxton RA, Sabatini DM. 2017. mTOR Signaling in Growth, Metabolism, and Disease. *Cell*
 649 168:960-976.
- 650 35. Besnard E, Hakre S, Kampmann M, Lim HW, Hosmane NN, Martin A, Bassik MC, Verschueren E,
 651 Battivelli E, Chan J, Svensson JP, Gramatica A, Conrad RJ, Ott M, Greene WC, Krogan NJ, Siliciano
 652 RF, Weissman JS, Verdin E. 2016. The mTOR Complex Controls HIV Latency. *Cell Host Microbe*
 653 20:785-797.
- 654 36. Jin S, Liao Q, Chen J, Zhang L, He Q, Zhu H, Zhang X, Xu J. 2018. TSC1 and DEPDC5 regulate
 655 HIV-1 latency through the mTOR signaling pathway. *Emerg Microbes Infect* 7:138.
- 656 37. Zhang J, Gao Z, Ye J. 2013. Phosphorylation and degradation of S6K1 (p70S6K1) in response to
 657 persistent JNK1 Activation. *Biochim Biophys Acta* 1832:1980-8.
- 658 38. Baxter AE, Niessl J, Fromentin R, Richard J, Porichis F, Charlebois R, Massanella M, Brassard N,
 659 Alsahafi N, Delgado GG, Routy JP, Walker BD, Finzi A, Chomont N, Kaufmann DE. 2016.
 660 Single-Cell Characterization of Viral Translation-Competent Reservoirs in HIV-Infected Individuals.
 661 *Cell Host Microbe* 20:368-380.
- 662 39. Alimonti JB, Ball TB, Fowke KR. 2003. Mechanisms of CD4+ T lymphocyte cell death in human
 663 immunodeficiency virus infection and AIDS. *J Gen Virol* 84:1649-1661.
- 664 40. Doitsh G, Greene WC. 2016. Dissecting How CD4 T Cells Are Lost During HIV Infection. *Cell Host*
 665 *Microbe* 19:280-91.
- 666 41. Mohammadi P, di Iulio J, Munoz M, Martinez R, Bartha I, Cavassini M, Thorball C, Fellay J,
 667 Beerenwinkel N, Ciuffi A, Telenti A. 2014. Dynamics of HIV latency and reactivation in a primary
 668 CD4+ T cell model. *PLoS Pathog* 10:e1004156.
- 669 42. Kuo HH, Ahmad R, Lee GQ, Gao C, Chen HR, Ouyang Z, Szucs MJ, Kim D, Tsibris A, Chun TW,
 670 Battivelli E, Verdin E, Rosenberg ES, Carr SA, Yu XG, Lichterfeld M. 2018. Anti-apoptotic Protein
 671 BIRC5 Maintains Survival of HIV-1-Infected CD4(+) T Cells. *Immunity* 48:1183-1194 e5.
- 672 43. Doehle BP, Hladik F, McNevin JP, McElrath MJ, Gale M, Jr. 2009. Human immunodeficiency virus
 673 type 1 mediates global disruption of innate antiviral signaling and immune defenses within infected
 674 cells. *J Virol* 83:10395-405.

- 675 44. Yan N, Regalado-Magdos AD, Stiggelbout B, Lee-Kirsch MA, Lieberman J. 2010. The cytosolic
676 exonuclease TREX1 inhibits the innate immune response to human immunodeficiency virus type 1.
677 *Nat Immunol* 11:1005-13.
- 678 45. Vermeire J, Roesch F, Sauter D, Rua R, Hotter D, Van Nuffel A, Vanderstraeten H, Naessens E,
679 Iannucci V, Landi A, Witkowski W, Baeyens A, Kirchhoff F, Verhasselt B. 2016. HIV Triggers a
680 cGAS-Dependent, Vpu- and Vpr-Regulated Type I Interferon Response in CD4(+) T Cells. *Cell Rep*
681 17:413-424.
- 682 46. O'Regan AW, Nau GJ, Chupp GL, Berman JS. 2000. Osteopontin (Eta-1) in cell-mediated immunity:
683 teaching an old dog new tricks. *Immunology Today* 21:475-478.
- 684 47. Burdo TH, Ellis RJ, Fox HS. 2008. Osteopontin is increased in HIV-associated dementia. *J Infect Dis*
685 198:715-22.
- 686 48. Joska JA, Combrinck M, Valcour VG, Hoare J, Leisegang F, Mahne AC, Myer L, Stein DJ. 2010.
687 Association between apolipoprotein E4 genotype and human immunodeficiency virus-associated
688 dementia in younger adults starting antiretroviral therapy in South Africa. *J Neurovirol* 16:377-83.
- 689 49. Andres MA, Feger U, Nath A, Munsaka S, Jiang CS, Chang L. 2011. APOE epsilon 4 allele and CSF
690 APOE on cognition in HIV-infected subjects. *J Neuroimmune Pharmacol* 6:389-98.
- 691 50. Chang L, Andres M, Sadino J, Jiang CS, Nakama H, Miller E, Ernst T. 2011. Impact of
692 apolipoprotein E epsilon4 and HIV on cognition and brain atrophy: antagonistic pleiotropy and
693 premature brain aging. *Neuroimage* 58:1017-27.
- 694 51. Morgan EE, Woods SP, Letendre SL, Franklin DR, Bloss C, Goate A, Heaton RK, Collier AC, Marra
695 CM, Gelman BB, McArthur JC, Morgello S, Simpson DM, McCutchan JA, Ellis RJ, Abramson I,
696 Gamst A, Fennema-Notestine C, Smith DM, Grant I, Vaida F, Clifford DB, Group CHATER. 2013.
697 Apolipoprotein E4 genotype does not increase risk of HIV-associated neurocognitive disorders. *J*
698 *Neurovirol* 19:150-6.
- 699 52. Hoare J, Westgarth-Taylor J, Fouche JP, Combrinck M, Spottiswoode B, Stein DJ, Joska JA. 2013.
700 Relationship between apolipoprotein E4 genotype and white matter integrity in HIV-positive young
701 adults in South Africa. *Eur Arch Psychiatry Clin Neurosci* 263:189-95.
- 702 53. Becker JT, Martinson JJ, Penugonda S, Kingsley L, Molsberry S, Reynolds S, Aronow A, Goodkin K,
703 Levine A, Martin E, Miller EN, Munro CA, Ragin A, Sacktor N. 2015. No association between
704 Apoepsilon4 alleles, HIV infection, age, neuropsychological outcome, or death. *J Neurovirol*
705 21:24-31.
- 706 54. Burt TD, Agan BK, Marconi VC, He W, Kulkarni H, Mold JE, Cavrois M, Huang Y, Mahley RW,
707 Dolan MJ, McCune JM, Ahuja SK. 2008. Apolipoprotein (apo) E4 enhances HIV-1 cell entry in vitro,

- 708 and the APOE epsilon4/epsilon4 genotype accelerates HIV disease progression. *Proc Natl Acad Sci*
 709 *U S A* 105:8718-23.
- 710 55. Dobson CB, Sales SD, Hoggard P, Wozniak MA, Crutcher KA. 2006. The receptor-binding region of
 711 human apolipoprotein E has direct anti-infective activity. *J Infect Dis* 193:442-50.
- 712 56. Kelly BA, Neil SJ, McKnight A, Santos JM, Sinnis P, Jack ER, Middleton DA, Dobson CB. 2007.
 713 Apolipoprotein E-derived antimicrobial peptide analogues with altered membrane affinity and
 714 increased potency and breadth of activity. *FEBS J* 274:4511-25.
- 715 57. Pollizzi KN, Powell JD. 2015. Regulation of T cells by mTOR: the known knowns and the known
 716 unknowns. *Trends Immunol* 36:13-20.
- 717 58. Heredia A, Le N, Gartenhaus RB, Sausville E, Medina-Moreno S, Zapata JC, Davis C, Gallo RC,
 718 Redfield RR. 2015. Targeting of mTOR catalytic site inhibits multiple steps of the HIV-1 lifecycle
 719 and suppresses HIV-1 viremia in humanized mice. *Proc Natl Acad Sci U S A* 112:9412-7.
- 720 59. Cinti A, Le Sage V, Milev MP, Valiente-Echeverria F, Crossie C, Miron MJ, Pante N, Olivier M,
 721 Moulant AJ. 2017. HIV-1 enhances mTORC1 activity and repositions lysosomes to the periphery by
 722 co-opting Rag GTPases. *Sci Rep* 7:5515.
- 723 60. van Montfort T, van der Sluis R, Darcis G, Beaty D, Groen K, Pasternak AO, Pollakis G, Vink M,
 724 Westerhout EM, Hamdi M, Bakker M, van der Putten B, Jurriaans S, Prins JH, Jeeninga R, Thomas
 725 AAM, Speijer D, Berkhout B. 2019. Dendritic cells potentially purge latent HIV-1 beyond
 726 TCR-stimulation, activating the PI3K-Akt-mTOR pathway. *EBioMedicine* 42:97-108.
- 727 61. Mbonye UR, Gokulrangan G, Datt M, Dobrowolski C, Cooper M, Chance MR, Karn J. 2013.
 728 Phosphorylation of CDK9 at Ser175 enhances HIV transcription and is a marker of activated P-TEFb
 729 in CD4(+) T lymphocytes. *PLoS Pathog* 9:e1003338.
- 730 62. Yoshinaga N, Shindo K, Matsui Y, Takiuchi Y, Fukuda H, Nagata K, Shirakawa K, Kobayashi M,
 731 Takeda S, Takaori-Kondo A. 2019. A screening for DNA damage response molecules that affect
 732 HIV-1 infection. *Biochem Biophys Res Commun* 513:93-98.
- 733 63. Murata M, Nishiyori-Sueki H, Kojima-Ishiyama M, Carninci P, Hayashizaki Y, Itoh M. 2014.
 734 Detecting expressed genes using CAGE. *Methods Mol Biol* 1164:67-85.
- 735 64. Kim D, Langmead B, Salzberg SL. 2015. HISAT: a fast spliced aligner with low memory
 736 requirements. *Nat Methods* 12:357-60.
- 737 65. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R,
 738 Genome Project Data Processing S. 2009. The Sequence Alignment/Map format and SAMtools.
 739 *Bioinformatics* 25:2078-9.
- 740 66. Kent WJ, Zweig AS, Barber G, Hinrichs AS, Karolchik D. 2010. BigWig and BigBed: enabling

- 741 browsing of large distributed datasets. *Bioinformatics* 26:2204-7.
- 742 67. Robinson MD, McCarthy DJ, Smyth GK. 2010. edgeR: a Bioconductor package for differential
743 expression analysis of digital gene expression data. *Bioinformatics* 26:139-40.
- 744 68. Benjamini Y, Hochberg Y. 1995. Controlling the False Discovery Rate: A Practical and Powerful
745 Approach to Multiple Testing. *Journal of the Royal Statistical Society: Series B (Methodological)*
746 57:289-300.
- 747 69. Robinson JT, Thorvaldsdottir H, Winckler W, Guttman M, Lander ES, Getz G, Mesirov JP. 2011.
748 Integrative genomics viewer. *Nat Biotechnol* 29:24-6.

749

750 **Figure Legends**

751 **FIG 1** Characterization of the HIV_{GKO} latency model. (A) Schema of the experimental
752 workflow. Briefly, Jurkat T cells were infected with HIV_{GKO} and 4 days later were analyzed
753 and sorted by FACS Aria II. Sorted cells were subjected to CAGE analysis and stimulation
754 with LRAs as indicated. (B) Representative FACS plot and gating at sorting. (C) At 4 days
755 post-infection, we sorted non-infected (N), latently infected (L) and productively infected
756 (P) cells, extracted total RNA and performed RT-qPCR. Unspliced (US), single spliced (SS)
757 and multiple spliced (MS) HIV-1 RNAs were quantified relative to cellular GAPDH and
758 presented as fold change relative to non-infected control (n = 3, mean ±SD). (D) Cells were
759 sorted as shown in (A). We extracted genomic DNA from each fraction and performed
760 Alu-based quantitative genomic PCR. The copy number of integrated provirus in each

761 fraction is presented relative to that of productively infected cells ($n = 3$, mean $\pm$ SD). (E) At
762 4 days post-infection, latently infected cells were sorted, cultured overnight and stimulated
763 with different LRAs for 24h before performing flow cytometry. Percentage of reactivated,
764 double positive (mKO2+, GFP+) cells is shown ($n = 2$, mean $\pm$ SEM).

765

766 **FIG 2** Overview of all detected CAGE tags and HIV derived CAGE tags. (A) Venn
767 diagram of the number of TSSs detected by CAGE in each cell fraction ($n = 2$, biologically
768 independent samples). Detected TSSs were defined as TSSs with an average CPM greater
769 than zero. TSSs that are annotated to specific gene promoters in the FANTOM5 database
770 are defined as annotated and the remaining TSSs are defined as non-annotated. (B)
771 Integrative Genomics Viewer (IGV) display of normalized mapped read counts (counts per
772 million; CPM) along the HIV_{GKO} genome for each fraction (N – non-infected; L – latently
773 infected; P – productively infected cells). The genome structure of HIV_{GKO} is shown at the
774 bottom. (C) Enlarged IGV view of the U3/R boundary in the 5' LTR and showing the
775 normalized mapped read counts in this region for each fraction. (D) Enlarged IGV view of

776 the EF1 α locus showing the normalized mapped read counts in this region for each
777 fraction.

778

779 **FIG 3** Significantly differentially expressed TSSs between latently infected and HIV_{GKO}
780 non-exposed Jurkat T cells are common in the other comparisons. (A) Number of the
781 significantly differentially expressed TSSs when comparing the cell fractions. (B) Venn
782 diagram of the number of significantly differentially expressed TSSs in each comparison.
783 (C and D) Volcano plots of differentially expressed TSSs between unexposed Jurkat cells
784 and non-infected cells (C) or latently infected cells (D) with log *p*-value and log fold
785 change graphed. Significantly differentially expressed TSSs that are common in all three
786 comparisons (J vs N, J vs L and J vs P) are shown in red. 679 and 343 TSSs that are
787 specifically significant in the comparison of J vs N and J vs L are shown in blue. (E)
788 Heatmap of gene ontology (GO) enrichment analysis of significantly differentially
789 expressed genes in each comparison. Significantly enriched GO terms (FDR < 0.05) in each
790 comparison are shown.

791

792 **FIG 4** Differential TSS expression in latently infected cells. (A) Cluster dendrogram of
793 CAGE results for non-infected (N), latently infected (L) and productively infected (P) cells,
794 using Spearman's rank-order correlation ($n = 2$, biologically independent samples). (B)
795 Number of significantly differentially expressed TSSs ($q < 0.05$) when comparing cell
796 fractions. (C and D) Volcano plots of differentially expressed TSSs between non-infected
797 and productively infected cells (C) or latently infected and productively infected cells (D)
798 with log p-value and log fold change graphed. Significantly differentially expressed TSSs
799 are shown in red. Arrows indicate genes associated with T-cell activation and apoptosis. (E)
800 Volcano plot of differentially expressed TSSs between non-infected and latently infected
801 cells with log q-value and log fold change graphed. Significantly differentially expressed
802 TSSs are shown in red. The top four significantly upregulated genes in non-infected cells
803 and the top two significantly upregulated TSSs in latently infected cells are indicated. (F)
804 Enrichment analysis of the upregulated genes in virus producing cells compared to
805 non-infected or latently infected cells. Representative GO terms in each comparison are
806 shown.

807

808 **FIG 5** Expression levels of interferon related genes by CAGE. Normalized expression
809 levels of genes involved in IFN response are shown. n.d.: not detected in CAGE; * $q < 0.05$

810

811 **FIG 6** Differentially expressed genes have an anti-viral property. (A) Heatmap of the 33
812 significantly ($q < 0.05$) differentially expressed TSSs between non-infected and latently
813 infected cells. (B) RT-qPCR of SPP1 in Jurkat T cells stably transduced with shRNA
814 against luciferase (non-target control) or SPP1 and treated with or without doxycycline for
815 4 days. Results are presented relative to expression in doxycycline untreated controls ($n = 2$,
816 mean $\pm$ SD, * $p < 0.05$, Student's t-test). (C) Percentage of latently infected (L) and
817 productively infected (P) cells after SPP1 knock down ($n = 2$, mean $\pm$ SEM). Jurkat T cells
818 stably transduced with shRNA against luciferase or SPP1 were treated with or without
819 doxycycline for 4 days, infected with HIV_{GKO} and infection rates were analyzed 2 days
820 post-infection (* $p < 0.05$, Student's t-test). (D) RT-qPCR of APOE in Jurkat T cells stably
821 transduced with shRNA against luciferase (non-target control) or APOE and treated with or
822 without doxycycline for 4 days. Results are presented relative to expressions in doxycycline
823 untreated controls ($n = 2$, mean $\pm$ SD, * $p < 0.05$, Student's t-test). (E) Percentage of latently

824 infected (L) and productively infected (P) cells after APOE knock down ($n = 2$, mean
825 $\pm$ SEM). Jurkat T cells stably transduced with shRNA against luciferase or APOE were
826 treated with or without doxycycline for 4 days, infected with HIV_{GKO} and infection rates
827 were analyzed 2 days post-infection ($*p < 0.05$, Student's t-test).

828

829 **FIG 7** Depletion of SPP1 or of APOE promotes viral replication in primary CD4⁺ T cells.

830 (A) RT-qPCR of SPP1 in primary CD4⁺ T cells transfected with non-target control siRNA,

831 siRNA against SPP1 #1 or #2. Results are presented relative to expression levels in

832 non-target controls ($n = 2$, mean $\pm$ SD, $*p < 0.05$, Student's t-test). (B) RT-qPCR of APOE

833 in primary CD4⁺ T cells transfected with non-target control siRNA, siRNA against APOE

834 #1 or #2. Results are presented relative to expression levels in non-target controls ($n = 2$,

835 mean $\pm$ SD, $*p < 0.05$, Student's t-test). (C) Percentage of latently infected (L) and

836 productively infected (P) cells after SPP1 knock down. Primary CD4⁺ T cells were

837 stimulated with anti-CD3/28 beads, then transduced with siRNA and infected with HIV_{GKO}.

838 Infection rates were analyzed at 2 days post-infection. Results from 2 donors are shown.

839 (D) Percentage of infected cells (latently and productively) after APOE knock down.

840 Primary CD4⁺ T cells were stimulated with anti-CD3/28 beads, then transduced with
841 siRNA and infected with HIV_{GKO}. Infection rates were analyzed at 2 days post-infection.
842 Results from 2 donors are shown. (E-F) Time course of late RT products when SPP1(E) or
843 APOE (F) were knocked down in primary CD4⁺ T cells. Primary CD4⁺ T cells were
844 stimulated with anti-CD3/28 beads, then transduced with siRNA against control or target
845 genes and infected with HIV_{GKO}. Late RT products at each time point (0, 4, 8, 12 and 24h)
846 were quantified by q-PCR. Results of donor 1 are presented as copy numbers per 1ng DNA
847 (n = 2, mean ±SD). (G-H) Quantitative PCR results of 2-LTR circles upon SPP1 (G) and
848 APOE (H) knock down. 2-LTR circles at 24h post-infection were quantified by q-PCR.
849 Results are presented as relative copy number to control (n = 2, mean ±SD). (I-J)
850 Quantitative PCR results of integrated HIV upon SPP1 (I) and APOE (J) knock down.
851 Integrated HIV at 24h post-infection was quantified by q-PCR. Results are presented as
852 copy number relative to control (n = 2, mean ±SD, **p* < 0.05, Student's t-test)

853

854 **FIG 8** Impact of the mTOR signaling pathway in establishing the latent reservoir. (A–C)
855 CAGE results at single gene level. The normalized expression levels of three significantly

856 differentially expressed genes ($*q < 0.05$) that belong to the mTOR signaling pathway are
857 shown. (D–F) Target gene immunoblotting results of Jurkat T cells stably transduced with
858 shRNA against luciferase (non-target control), MLST8 (D), EIF4EBP1 (E) or RPS6 (F)
859 treated with or without doxycycline for 4 days. (G–I) Percentages of latently infected (L)
860 and productively infected (P) cells after knock down of each gene shown in (A–C) ($n = 2$,
861 mean $\pm$ SEM). Jurkat T cells stably transduced with shRNA against luciferase (non-target
862 control), MLST8 (G), EIF4EBP1 (H) or RPS6 (I) were treated with or without doxycycline
863 for 4 days, infected with HIV_{GKO} and infection rates were analyzed 4 days post-infection
864 ($*p < 0.05$, Student's t-test). (J) mTOR, S6K and phosphorylated mTOR (p-mTOR, S2448)
865 and S6K (p-S6K, T389) immunoblotting results of cells treated as in (G–I). (K) p-S6K
866 (T389) and S6K immunoblotting results of sorted HIV_{GKO} infected Jurkat T cells. Tubulin
867 was used as a loading control in images (D–F, J and K). (L) and (M) Jurkat T cells were
868 infected with HIV_{GKO}, incubated for 24h, then treated with specific S6K inhibitors
869 (LY2584702 (L) and PF-4708671 (M)) and the percentage of latently and productively
870 infected cells assessed at 4 days post-infection ($*p < 0.05$, Student's t-test).

871

872 **FIG 9** S6K inhibition promotes establishment of latency in primary CD4⁺ T cells. Primary
873 CD4⁺ T cells were stimulated with anti-CD3/28 beads for 3 days and infected with HIV_{GKO}.
874 At 24 hours post-infection, CD4⁺ T cells were treated with specific S6K inhibitors
875 (LY2584702 or PF-470861) and the percentage of latently infected cells was evaluated at 4
876 days post-infection. Results from donor 1 (A) and donor 2 (B) are shown.

877

878

FIG 1

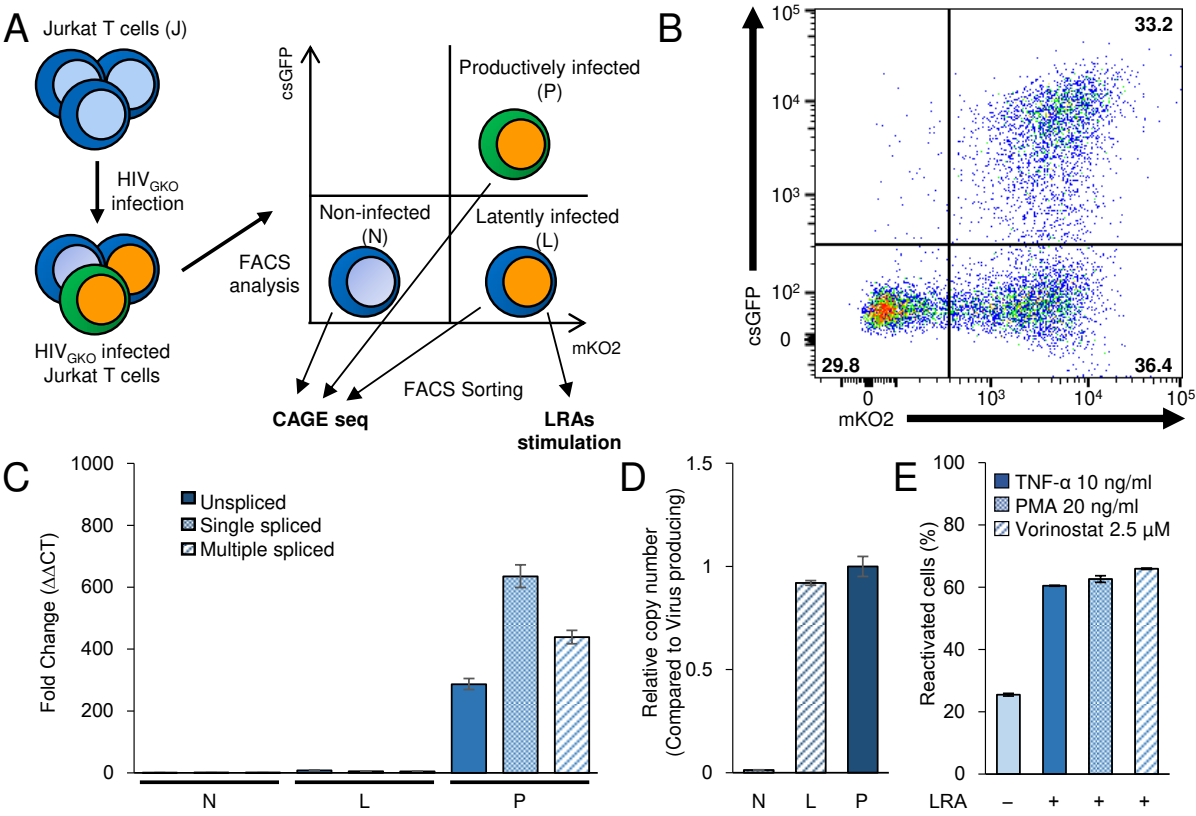


FIG 2

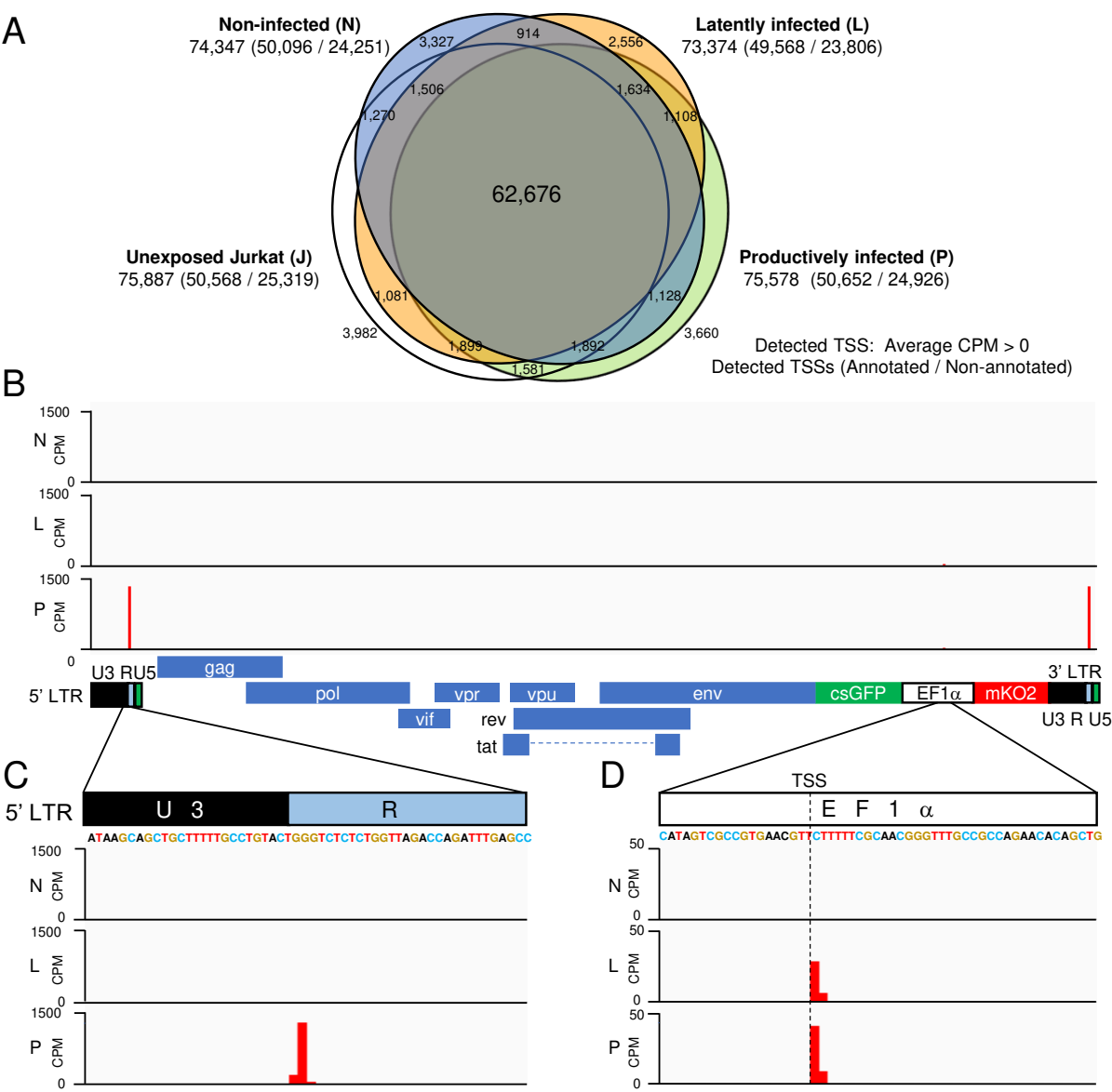


FIG 3

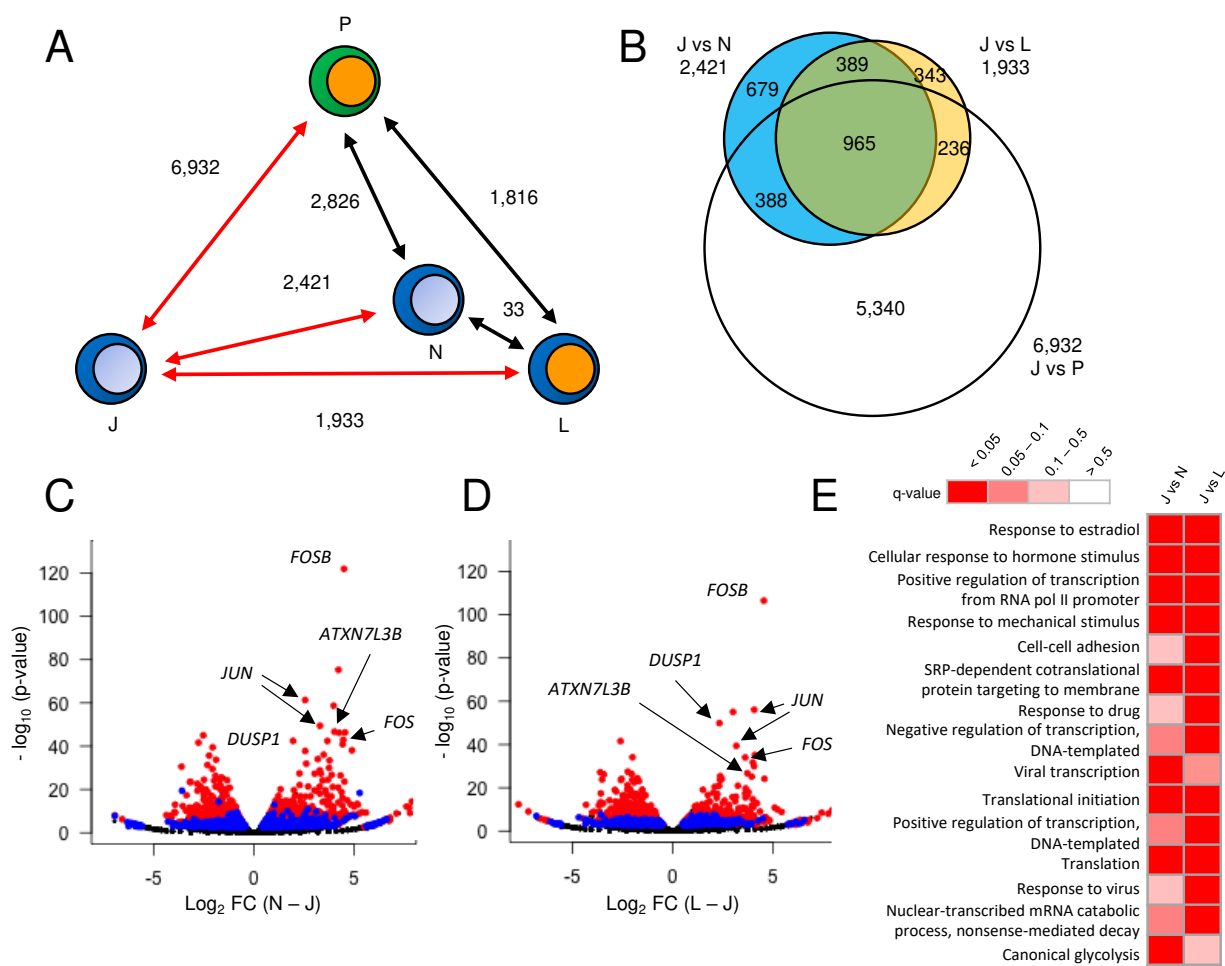


FIG 4

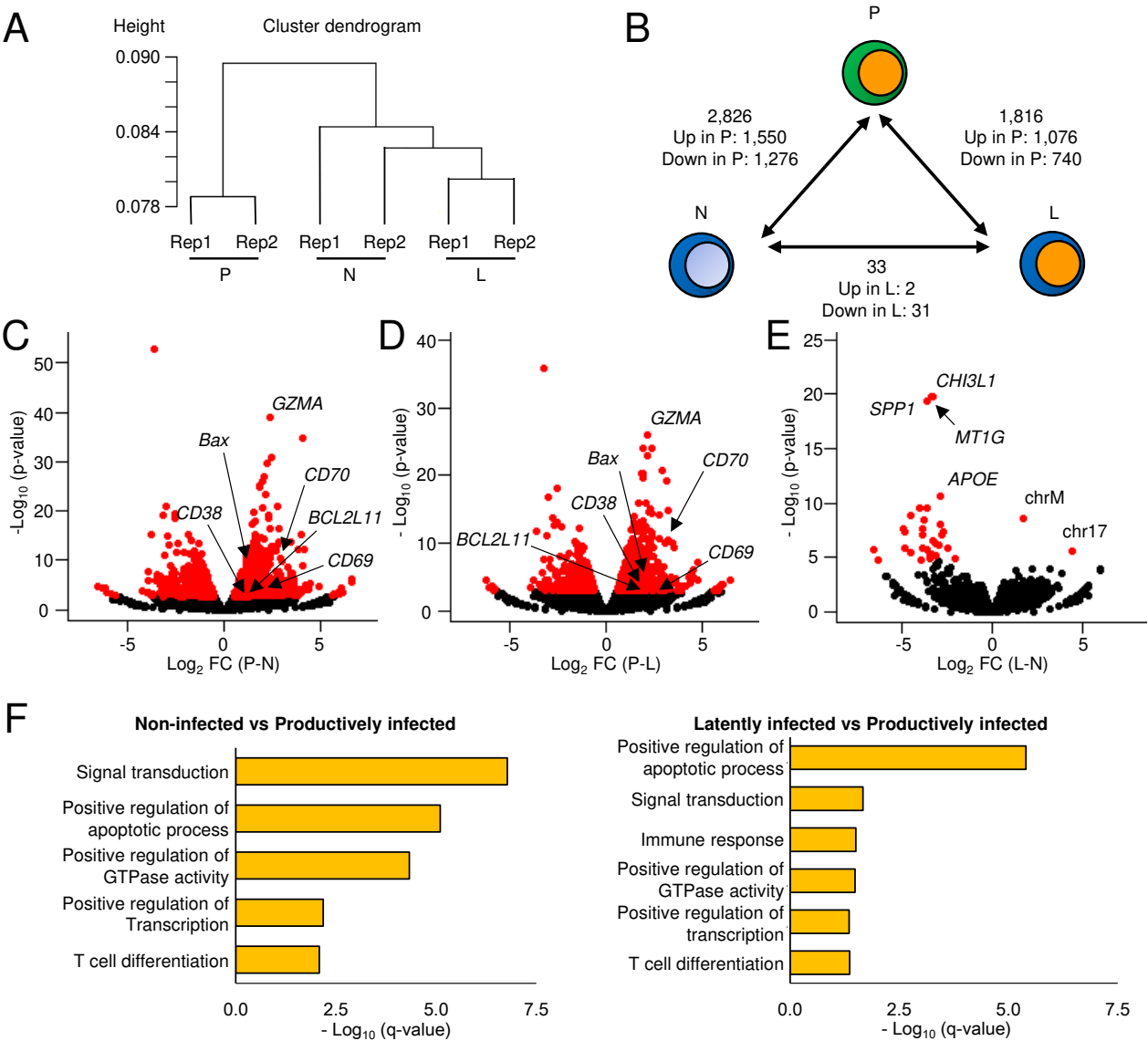


FIG 5

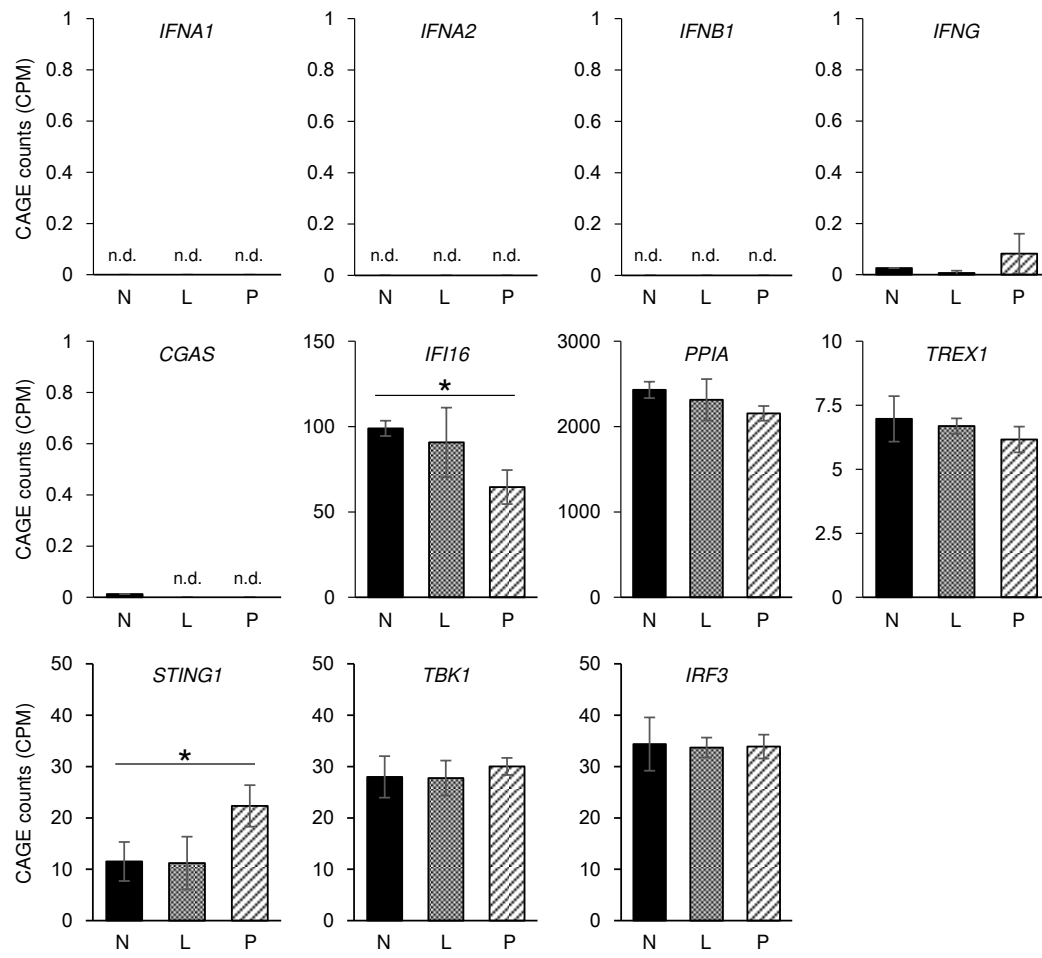


FIG 6

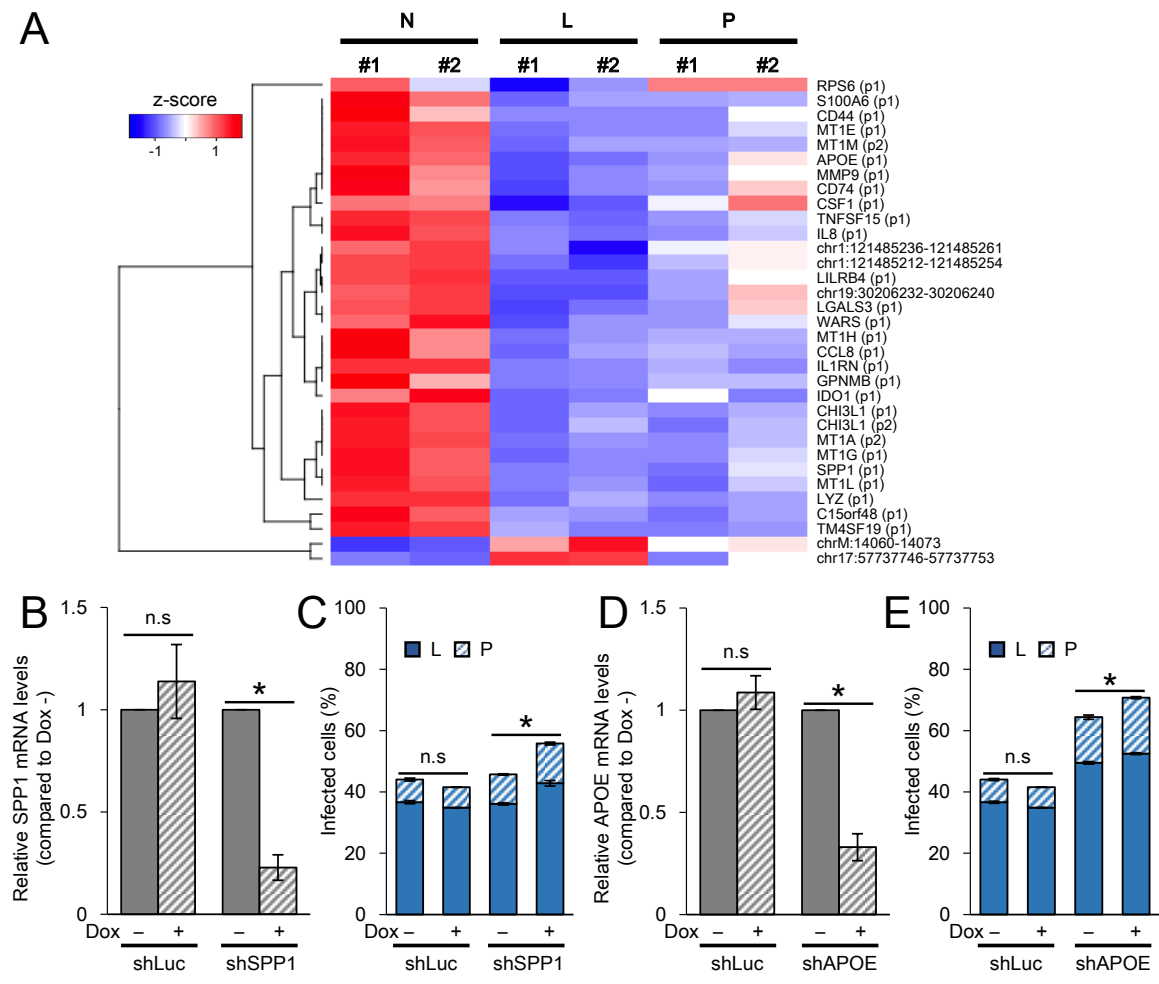


FIG 7

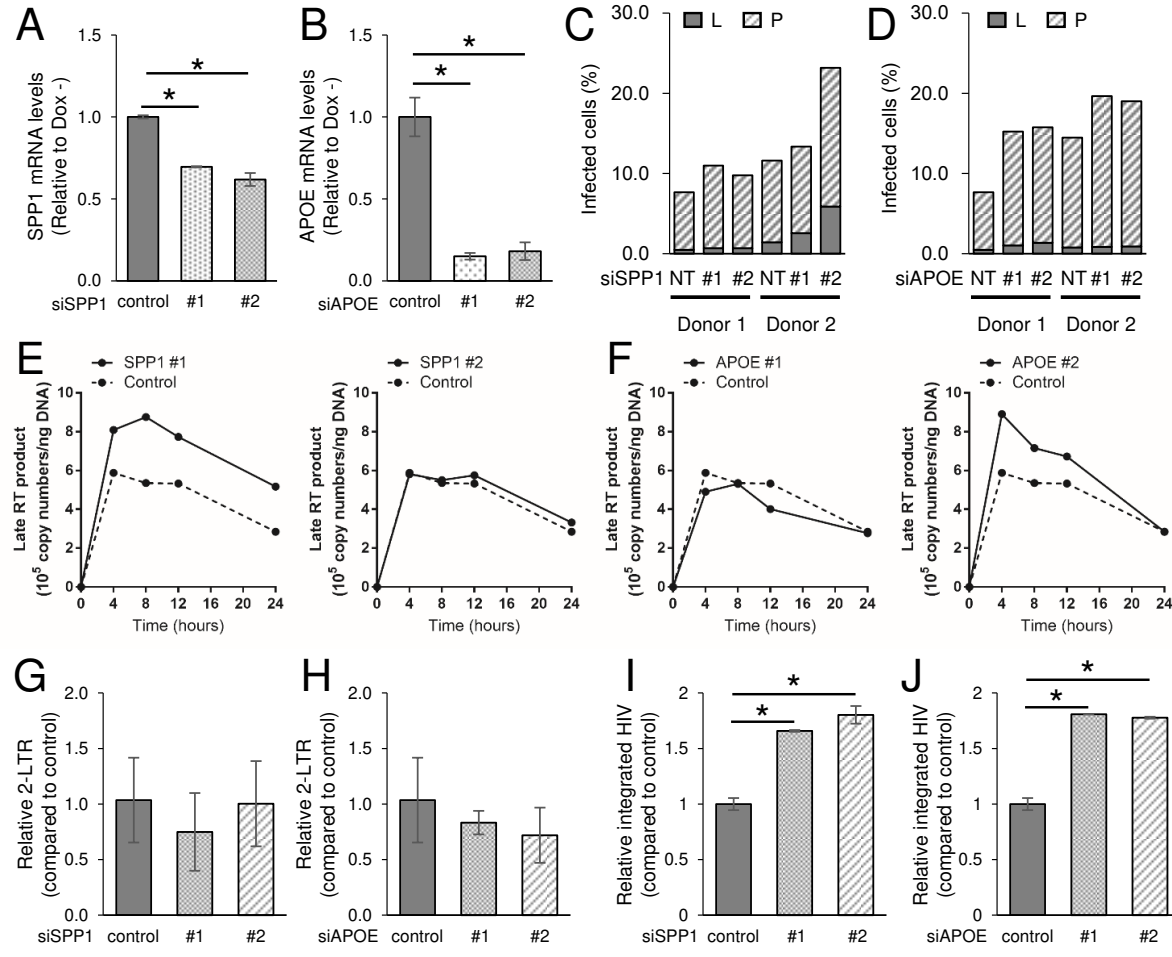


FIG 8

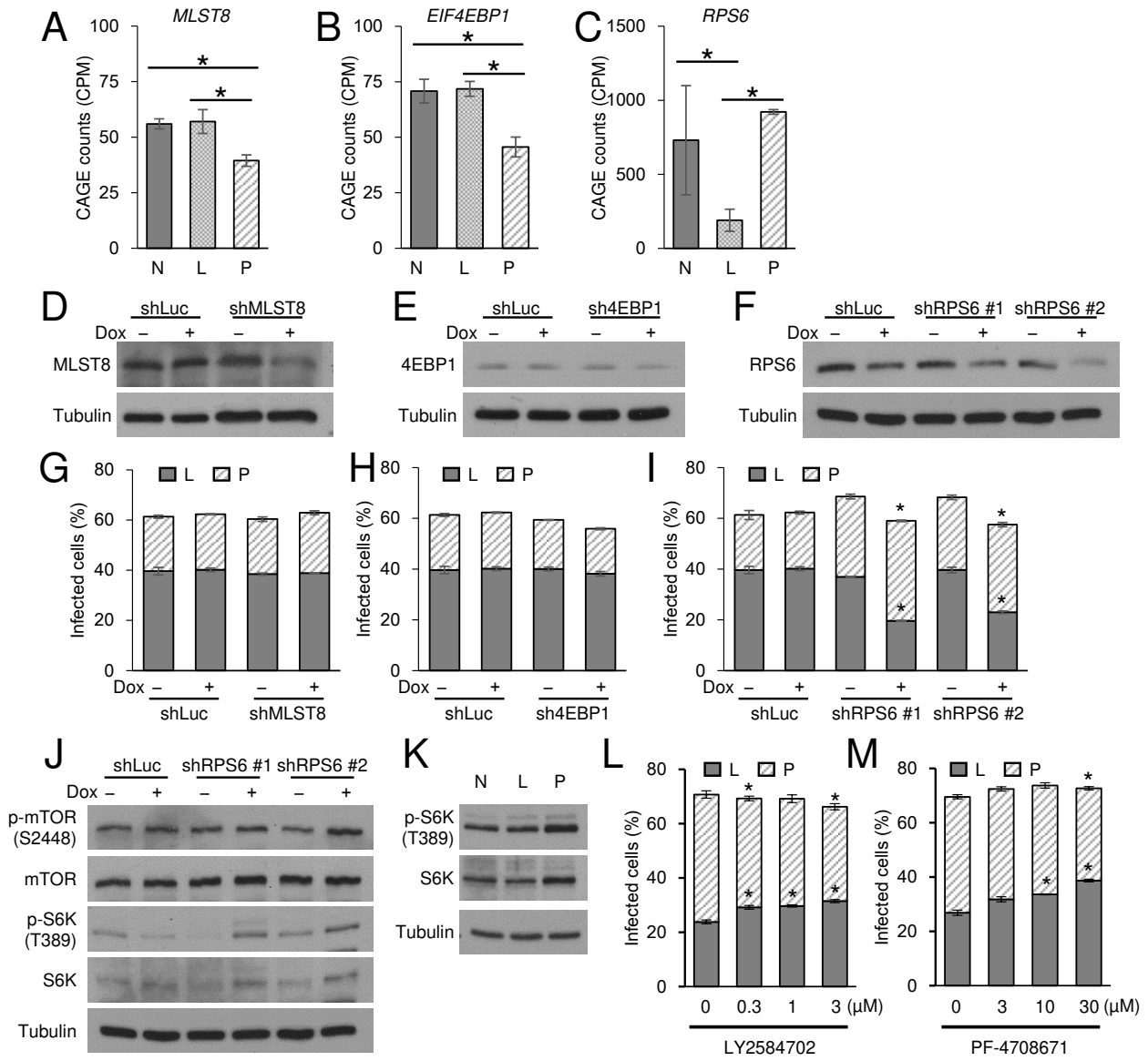


FIG 9

