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1 **The XPB subunit of the TFIIH complex plays a critical role in HIV-1 transcription**
2 **and XPB inhibition by Spironolactone prevents HIV-1 reactivation from latency**

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15
16 **Running title: Spironolactone blocks HIV-1 reactivation from latency**

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23

24 **ABSTRACT**

25 HIV transcription requires assembly of cellular transcription factors at the HIV-
26 1promoter. The TFIID general transcription factor facilitates transcription initiation by
27 opening the DNA strands around the transcription start site and phosphorylating the C-
28 terminal domain for RNA polymerase II (RNAPII) for activation. Spironolactone (SP), an
29 FDA approved aldosterone antagonist, triggers the proteasomal degradation of the XPB
30 subunit of TFIID, and concurrently suppresses acute HIV infection *in vitro*. Here we
31 investigated SP as a possible block-and-lock agent for a functional cure aimed at the
32 transcriptional silencing of the viral reservoir. The long-term activity of SP was
33 investigated in primary and cell line models of HIV-1 latency and reactivation. We show
34 that SP rapidly inhibits HIV-1 transcription by reducing RNAPII recruitment to the HIV-1
35 genome. shRNA knockdown of XPB confirmed XPB degradation as the mechanism of
36 action. Unfortunately, long-term pre-treatment with SP does not result in epigenetic
37 suppression of HIV upon SP treatment interruption, since virus rapidly rebounds when
38 XPB reemerges; however, SP alone without ART maintains the transcriptional
39 suppression. Importantly, SP inhibits HIV reactivation from latency in both cell line
40 models and resting CD4⁺T cells isolated from aviremic infected individuals upon cell
41 stimulation with latency reversing agents. Furthermore, long-term treatment with
42 concentrations of SP that potently degrade XPB does not lead to global dysregulation of
43 cellular mRNA expression. Overall, these results suggest that XPB plays a key role in
44 HIV transcriptional regulation and XPB degradation by SP strengthens the potential of
45 HIV transcriptional inhibitors in block-and-lock HIV cure approaches.

46

47 **IMPORTANCE**

48 Antiretroviral therapy (ART) effectively reduces an individual's HIV loads to below the
49 detection limit, nevertheless rapid viral rebound immediately ensues upon treatment
50 interruption. Furthermore, virally suppressed individuals experience chronic immune
51 activation from ongoing low-level virus expression. Thus, the importance of identifying
52 novel therapeutics to explore in block-and-lock HIV functional cure approaches, aimed
53 at the transcriptional and epigenetic silencing of the viral reservoir to block reactivation
54 from latency. We investigated the potential of repurposing the FDA-approved
55 spironolactone (SP), as one such drug. SP treatment rapidly degrades a host
56 transcription factor subunit, XPB, inhibiting HIV transcription and blocking reactivation
57 from latency. Long-term SP treatment does not affect cellular viability, cell cycle
58 progression or global cellular transcription. SP alone blocks HIV transcription in the
59 absence of ART but does not delay rebound upon drug removal as XPB rapidly
60 reemerges. This study highlights XPB as a novel drug target in block-and-lock
61 therapeutic approaches.

62

63

64 INTRODUCTION

65 Upon HIV-1 integration into the host genome the provirus becomes transcriptionally
66 silent as the cell returns to a quiescent state (1). Despite effective antiretroviral therapy
67 (ART) that suppresses HIV-1 replication to below the detection limit, these latent
68 proviruses can reinitiate viral production upon cell stimulation or treatment interruption
69 (2–4). The total elimination of this latent reservoir has become a major goal of HIV-1
70 eradication strategies and interventions involving latency modifying agents, immune
71 therapies, or gene and cell-based therapies are being investigated (5–7). More recently
72 though, HIV-1 remission or a functional cure have been explored (8, 9). A functional
73 cure entails either null or sufficiently low viral replication that is controlled by the immune
74 system in the absence of ART, while proviral DNA remains detectable. This would allow
75 the prospect of functionally cured individuals maintaining undetectable viral loads in the
76 absence of ART, without risk of onward transmission and no ongoing immunological
77 damage that is associated with HIV infection (10–12). One such functional cure
78 approach has been branded the block-and-lock approach, which aims at the
79 transcriptional silencing of the viral reservoir (5). There are unfortunately no clinically
80 available HIV-1 specific transcriptional inhibitors.

81 Transcription at the HIV-1 promoter begins with the assembly of the pre-initiation
82 complex (PIC). The 5' long terminal repeat (LTR), which serves as the viral promoter,
83 contains binding sites for multiple transcription factors (TFs) which bind sequentially to
84 form the PIC. These include NF- κ B, Sp1, TATA-binding protein (TBP) (as part of the
85 general transcription factor TFIID),TFIIA, TFIIB and TFIIF, which together direct RNA
86 polymerase II (RNAPII) binding to form a stable promoter complex (13–15). The PIC is

87 completed by the addition of the TFIIE heterodimer and TFIIH (15). TFIIH is a ten-
88 protein complex consisting of a core (XPB, XPD, p62, p52, p44, p34 and p8) and cyclin-
89 dependent kinase (CDK)-activating kinase (CAK) subcomplex (CDK7, Cyclin H and
90 MAT1) (16). TFIIH plays a crucial role in the initiation of transcription, but also in
91 nucleotide excision repair, where bulky adducts are removed from damaged DNA (17).
92 In transcription, once the PIC has assembled, the XPB subunit functions as a DNA
93 translocase, feeding the DNA into the RNAPII cleft, introducing negative supercoiling
94 and initiating the formation of the transcription bubble (16, 18, 19). The CDK7 subunit is
95 responsible for the initial activating phosphorylation of serine 5 in the heptapeptide
96 repeats on the RNAPII (CTD) tail (pSer5) (20–22). Activated RNAPII clears the
97 promoter, but rapidly stalls after transcribing the first ~59 nucleotides downstream of the
98 transcription start site (TSS), which comprises a secondary structure termed the
99 transactivation response element (TAR) (23). RNAPII stalls for a few reasons, namely
100 its association with negative elongation factors, NELF and DSIF, as well as the
101 presence of Nucleosome-1 (Nuc-1) downstream from the TSS that occlude efficient
102 elongation (24–26). The high affinity interaction of the viral protein Tat, which is
103 produced from rare full-length transcripts, with the TAR RNA allows recruitment of the
104 positive elongation factor, P-TEFb, consisting of CyclinT1 and CDK9 (24, 25, 27–33).
105 CDK9 phosphorylation of NELF (leading to detachment from RNAPII), DSIF (converts to
106 an elongation factor), as well as Ser2 on the RNAPII CTD allows transcription to
107 proceed (34, 35). Tat plays a fundamental role in HIV transcription and is key for the
108 rapid and robust increase in full-length viral transcripts from the HIV genome upon
109 reactivation from latency (28, 36–39). The proof-of-concept for the block-and-lock

110 approach was shown with the small molecule inhibitor of Tat, didehydro cortistatin A
111 (dCA) in CD4⁺ T cells isolated from infected individuals and in the humanized mouse
112 model of HIV latency (40–42). dCA directly interacts with the basic domain of Tat,
113 blocking the interaction of Tat with TAR and preventing transcriptional elongation (40).
114 As such, the long-term treatment of cells with dCA restricts RNAPII recruitment to the
115 promoter and over time epigenetic marks are deposited at the HIV-1 promoter,
116 promoting silencing and blocking reactivation from latency (41, 43, 44).

117 In search for novel molecules that regulate HIV transcription to increase our
118 portfolio of block-and-lock approaches, we studied the action of spironolactone (SP). SP
119 is a potassium-sparing diuretic, an aldosterone antagonist that blocks binding to the
120 mineralocorticoid receptor (MR), and has been approved since 1959 for the treatment of
121 a range of conditions, including hypertension and heart failure (45). An off-target effect
122 of SP is the proteasomal degradation of the ATP-dependent helicase, XPB subunit of
123 the transcription factor TFIIH complex (46–49). XPB degradation is dependent on the
124 26S proteasome and ubiquitinating enzymes (46). SP triggers XPB Ser90
125 phosphorylation by the CDK7 subunit of TFIIH leading to SCF^{FBXL18} E3 ligase-mediated
126 ubiquitination and proteasomal degradation (50). Despite XPB's critical role in
127 transcription initiation (14, 16), its degradation was reported not to affect global
128 transcription (51, 52). However, subsequent studies showed transcriptional inhibition of
129 genes highly dependent on NF- κ B (47, 51). The two binding sites for NF- κ B at the HIV-
130 1 LTR allow NF- κ B control of HIV-1 reemergence from latency (53). Lacombe *et al.*
131 previously demonstrated that XPB degradation, by short-term treatment of cells with SP
132 inhibits acute HIV-1 and HIV-2 transcription (48).

133 Here, we explored the potential of SP as a block-and-lock agent, through long-
134 term treatment of latently infected cells and resting CD4⁺ T cells explanted from infected
135 individuals. We demonstrated the ability of SP to inhibit HIV-1 transcription in latently
136 infected cells and suppress reactivation from latency by potent LRAs, which correlated
137 with loss of RNAPII recruitment to the HIV-1 promoter and genome, without damaging
138 cellular effects. We confirmed the activity of SP was mediated by XPB via knockdown
139 studies and is independent of the Tat-TAR interaction. Altogether, our results suggest
140 that XPB plays a key role in HIV-1 transcriptional activation and its inhibition by SP
141 blocks HIV-1 expression.

142

143 RESULTS

144

145 Treatment of latently infected cell lines with SP inhibits transcription of HIV-1.

146 SP has been previously shown to block acute HIV infection of CD4⁺ T cells (48).
147 Here to explore whether SP could be used in block-and-lock approaches to an HIV cure
148 we investigated its ability to inhibit transcription from cell line models of latency. We
149 studied SP activity in two established cell line models of latency, OM-10.1 and ACH-2
150 cells, in the presence of ART. OM-10.1 cells are a promyelocytic line derived from HL-
151 60 cells that survived an acute HIV-1 infection. Each cell contains a single integrated,
152 full length provirus (54). SP treatment of these cells resulted in a dose-dependent
153 reduction of HIV-1 capsid p24 in the supernatant, with an IC₅₀ of 1.77 μM (Fig. 1A). This
154 inhibition correlated with a dose-dependent degradation of the cellular protein XPB (Fig.
155 1B). Eplerenone (EPL), a more selective mineralocorticoid receptor (MR) antagonist
156 analog of SP, does not lead to XPB degradation (46, 47, 51, 55). As previously reported

157 (48), no inhibition of HIV-1 was observed with increasing concentrations of EPL (Fig.
158 1C), suggesting the mechanism of HIV-1 inhibition is independent of MR antagonism.
159 No cytotoxicity was observed with up to 50 μ M SP or EPL following a 72 h incubation
160 (Fig. 1D).

161 Next, we evaluated the long-term effects of SP treatment. OM-10.1 cells were
162 treated with 10 μ M SP, 10 μ M EPL or DMSO for 190 days (Fig. 1E), and capsid p24
163 released into the supernatant was quantified by p24 ELISA. In OM-10.1 cells, SP rapidly
164 inhibits viral production by approximately 2.5 logs, with capsid p24 levels stabilizing
165 around the limit of detection (3 pg/ml). As expected, a reduction in the HIV-1 mRNA
166 expression was observed in parallel (Fig. 1F, red line). The mRNA levels of the XPB
167 gene, *ERCC3*, were either unaffected or slightly elevated by SP treatment (Fig. 1F,
168 brown line). This confirms previous reports that degradation of XPB by SP is mediated
169 by the 26S proteasome and not by reduced XPB mRNA expression (46). Of note, the
170 half-life of SP in long-term culture of OM-10.1 cells with ART was determined by LC-
171 MS/MS to be 2.3 (+/- 0.2) days and cells were split and treated every three days. All
172 experiments were performed in the presence of ART unless otherwise stated. Of note,
173 without ART the half-life is 2.9 (+/- 1.7) days). Altogether these results suggest that, in
174 OM-10.1 cells, SP treatment combined with ART over time inhibits HIV-1 transcription to
175 very low, frequently undetectable levels without obvious cytotoxic effects in the cells.

176 We next assessed the activity of SP in the ACH-2 cell line, a T cell clone
177 containing a single integrated provirus (56, 57), which harbors a C37T mutation TAR
178 such that the Tat-TAR feedback loop is defective resulting in low transcription rates (58,
179 59). SP treatment of these cells resulted in a similar dose-dependent reduction of HIV-1

180 capsid p24 in the supernatant, with an IC_{50} of 1.95 μ M (Fig. 2A), which correlated with a
181 dose-dependent degradation of XPB (Fig. 2B). Again, EPL was inactive against HIV
182 (Fig. 2C). Cytotoxicity was not observed up to 20 μ M of SP for a 72 h period, nor up to
183 50 μ M with EPL (Fig. 2D). The long-term treatment of ACH-2 cells inhibited viral
184 production by 1-1.5 logs, with capsid production stabilizing around 20-100 pg/ml (Fig.
185 2E). As expected, a reduction in cell associated HIV RNA was also observed (Fig. 2E),
186 while levels of XPB mRNA were either unaffected or slightly elevated (Fig. 2F). No
187 difference in cell viability or cell number was observed over time (Figure 2G).
188 Differences in the magnitude of SP activity on HIV-1 p24 production between OM-10.1
189 and ACH-2 cells may be related to the level of XPB degradation in these cell lines, 95%
190 in OM-10.1 cells as compared to 81% in ACH-2 cells (Fig. 1B and 2B). Given that SP
191 inhibits the transcription from proviruses with an incompetent Tat-TAR feedback loop,
192 as is the case of ACH-2 cells, the mechanism of action of SP is likely independent of the
193 Tat-TAR interaction.

194 We also investigated the potential of SP to inhibit SIV_{mac239} in HUT-78 cells.
195 Potent suppression of SIV replication with SP, but not EPL or DMSO was observed over
196 a period of 12 days (Fig. 2G). This was accompanied by degradation of XPB by SP
197 treatment as measured by Western blot on day 12 (Fig. 2H, inset panel). A rapid decline
198 in cellular viability, due to cytotoxic effects of virus replication, was observed in DMSO
199 and EPL treated cells where virus replication was allowed to proceed, whilst the viability
200 of cells treated with 10 μ M SP was not adversely affected (Fig. 2I). These results
201 suggest that SP may be used in latency studies in non-human primates infected with
202 SIV.

203 In sum, these results suggest that SP inhibits HIV-1 transcription in established
204 cell line models of latency.

205

206

207 **RNA silencing of the XPB gene inhibits HIV-1 transcription.**

208 To confirm that HIV-1 inhibition by SP was truly dependent on XPB degradation, and
209 not the result of an off-target effect, we knocked down the XPB gene, *ERCC3*, by
210 shRNA and assessed viral replication. OM-10.1 cells were transduced with a lentiviral
211 vector carrying either an *ERCC3* specific shRNA or an shRNA targeting the GFP gene
212 as control (since these cells do not contain GFP). In parallel, cells transduced with
213 shGFP were treated with the $IC_{50} = 1.8 \mu\text{M}$ and $IC_{90} = 6.3 \mu\text{M}$ concentrations of SP, as
214 well as $10 \mu\text{M}$ SP, to compare XPB levels and HIV replication. After 48 h of culture, cells
215 were placed back on ART and selected with puromycin to enrich for cells containing the
216 shRNA. After 10 days of puromycin selection, HIV-1 capsid production in the
217 supernatant was measured by p24 ELISA, and cells were collected for RNA and
218 Western blot analysis.

219 A knockdown efficiency of 70-80% at both the protein and RNA level (Fig. 3A-B)
220 was achieved with the shRNA targeting *ERCC3*, while well tolerated by cells (Fig. 3C).
221 Notably, SP treatment resulted in a slight upregulation of *ERCC3* mRNA (Fig. 3B), as
222 previously observed (Fig. 1F), which we suspect is a cellular compensatory mechanism
223 for the loss of XPB protein. When compared to cells transduced with the shGFP control,
224 the *ERCC3* knockdown resulted in suppression of HIV *gag* mRNA and p24 production
225 to levels between the IC_{50} and IC_{90} concentrations of SP (Fig. 3D-E).

226 Importantly, when we compared expression of additional TFIIH components, we
227 observed substantial downregulation of XPD, CDK7 and cyclin H with SP treatment
228 (Fig. 3F). With *ERCC3* knock down, despite XPB downregulation similar to the IC₅₀ and
229 IC₉₀ concentrations of SP, XPD was not downregulated (Fig. 3F). However, although
230 not to the same degree as SP, a loss of CDK7 and Cyclin H were still detected (Fig.
231 3F).

232 Altogether our results strongly support that the inhibition of HIV-1 transcription
233 observed with SP treatment is the result of XPB degradation.

234

235 **Treatment of latently infected cells with SP reduces HIV-1 reactivation from**
236 **latency.**

237 We next investigated whether SP treatment could block the reactivation of latent HIV-1
238 proviruses when cells were exposed to LRAs. The specific LRAs and doses chosen to
239 reactivate each cell model were selected based on the ability to potently stimulate HIV-1
240 replication, with minimal toxicity. Prostratin, phorbol myristate acetate (PMA) and TNF- α
241 activate transcription by induction of NF- κ B, while the histone deacetylase (HDAC)
242 inhibitor suberoylanilide hydroxamic acid (SAHA) promotes chromatin acetylation,
243 namely at nucleosome-1 on the HIV-1 LTR (60–63).

244 OM-10.1 and ACH-2 cells were treated for 90 and 15 days, respectively, with 10
245 μ M SP or DMSO and the levels of HIV-1 capsid production were monitored over time by
246 p24 ELISA (Fig 4A-B). Cell number and viability remained stable over time (as in Figure
247 1 and 2). Once HIV levels had stabilized at maximal inhibition, the cells were treated
248 with a panel of LRAs in the presence of ART and either 10 μ M SP or DMSO. After an 8

249 h incubation period cells were collected and viral transcription assessed by RT-qPCR to
250 HIV *nef* mRNA. Robust reactivation was observed in DMSO+ART treated OM-10.1 and
251 ACH-2 cells (Fig. 4C-D, blue bars) with all LRAs tested. While some reactivation of HIV
252 in SP treated OM-10.1 and ACH-2 cells was observed in the presence of LRAs, these
253 were non-statistically significant (Fig. 6C-D, red bars). Similar results were observed in
254 the Jurkat latency models (J-Lat A1 and A2) (data not shown).

255 Next, we assessed the activity of SP in primary cells from ART-adherent
256 individuals living with HIV. Resting CD4⁺ T cells were isolated from peripheral blood
257 mononuclear cells (PBMCs) from seven individuals with undetectable HIV viral loads,
258 adherent to ART for at least one year with no viral load “blips” in the last six months.
259 These cells were treated for 24 h with either 10 μM SP or vehicle only in the presence of
260 the entry inhibitor T20, to prevent novel infections. In the final 6 h of treatment, cells
261 were challenged with PMA/ionomycin for T cell activation. SP treatment reduced HIV-1
262 reactivation an average of 1.4 log (27.6-fold) measured by RT-qPCR per million cells
263 (Fig. 4E). The expression of a housekeeping gene *POLR2A* was also measured and
264 showed modest, yet significantly increased levels (an average of 0.4 log, 2.5-fold) (Fig.
265 4F) (64). In sum, this demonstrates that SP inhibits reactivation from latency in primary
266 CD4⁺ T cells explanted from virally suppressed individuals.

267 In sum, these results show that treatment of latently infected cell lines, and ex
268 vivo stimulated primary CD4⁺T cells, with 10 μM SP dramatically reduces reactivation of
269 HIV-1 from latency when exposed to potent LRAs.

270

271 **SP treatment alone blocks viral reactivation; however, long-term SP treatment**
272 **does not prevent viral rebound upon treatment interruption.** The long term
273 treatment of HIV-1 infected cells with the Tat inhibitor dCA results in the accumulation of
274 epigenetic marks at the HIV-1 promoter, that render reactivation from latency very
275 difficult to occur upon treatment interruption or stimulation with LRAs (41, 43, 44). Thus,
276 we investigated whether SP treatment over time would lead to similar effects. First, we
277 tested whether 10 μ M SP alone was sufficient to maintain HIV transcription suppression
278 in the absence of ART. As such, we washed off ART on day 69 from the OM-10.1 cell
279 culture (three independent experiments) and measured viral p24 production in the
280 supernatant (Fig. 5A, black arrows). A non-significant increase in HIV p24 production
281 was observed in DMSO treated cells in the absence of ART (Fig. 8A, light blue line),
282 while no such increase was observed in SP treated cells (Fig. 5A, orange line),
283 suggesting SP treatment alone is able to block HIV replication in the absence of ART.
284 Next, we assessed whether pre-treatment with SP would delay HIV rebound upon all
285 treatment interruption, as such on day 78, DMSO and SP were washed off and cells
286 maintained on ART alone (Fig. 5B, black arrow). As expected, HIV p24 levels remained
287 stable in cells pre-treated with DMSO; however, within 12 days a rapid increase in virus
288 replication back to DMSO levels was observed in cells pre-treated with SP (Fig. 8B
289 orange line). Concomitantly, within 3 days of treatment interruption, we observed a rapid
290 rebound in XPB protein levels, as well as other TFIIH components (XPD, CDK7 and
291 Cyclin H) (Fig. 8C). In fact, XPB levels rebounded above the level of the DMSO control
292 and remained elevated for at least 9 days post treatment interruption (Fig. 8C, orange
293 lanes vs light blue lanes).

294 While pre-treatment with SP did not result in long-lasting epigenetic control of
295 viral transcription upon treatment interruption, SP treatment alone seems sufficient to
296 maintain HIV transcriptional inhibition in the absence of ART. These important results
297 suggest that SP blocks HIV transcription as a single-therapy and holds great potential to
298 be used in combination with other latency-promoting agents (LPAs), with the goal of
299 establishing an epigenetic lock on the HIV genome.

300

301 **SP treatment reduces RNAPII recruitment to the HIV-1 genome.**

302 To confirm that transcriptional inhibition is the mechanism of action of SP against HIV,
303 we investigated RNAPII recruitment to the HIV-1 genome. As such, we performed
304 chromatin immunoprecipitation (ChIP) to RNAPII followed by RT-qPCR using primers
305 recognizing the HIV-1 genome. We sought to identify differences in occupancy of total
306 as well as phosphorylated Ser5 or Ser2 RNAPII (pSer5, pSer2 RNAPII).

307 Three independent long-term treatments (~70 days) of OM-10.1 cells with
308 DMSO+ART or SP+ART (see Fig. 4A) were treated or not with 1 μ M prostratin for 8 h,
309 resulting in mean 4.3-fold increase in p24 production and a mean 147-fold increase in
310 HIV mRNA (data not shown) in DMSO+ART treated cells, with no detectable changes in
311 p24 production in SP treated cells observed (but a mean 5.3-fold increase in HIV
312 mRNA) (data not shown). These cells were crosslinked and prepared for RNAPII ChIP.
313 Without prostratin, with or without SP, a small peak of RNAPII was observed at the HIV
314 promoter, with very little occupancy throughout the genome (Fig. 6A). Modest
315 reductions in occupancy of total, pSer5 and pSer2 RNAPII in unstimulated SP+ART
316 treated cells were observed compared DMSO+ART control (Fig. 6B-C, red vs dark blue

317 lines). Stimulation with prostratin led to a significant increase of total RNAPII occupancy
318 in DMSO+ART treated cells, with a peak at the promoter and substantial occupancy
319 throughout the genome (Fig. 6A, light blue line). As expected, a strong peak of pSer5
320 RNAPII was observed at the HIV promoter in DMSO+ART treated cells stimulated with
321 prostratin, with a slight reduction in the gene body (Fig. 6B, light blue line). A peak of
322 pSer2 RNAPII was observed at the HIV promoter of DMSO+ART treated cells
323 stimulated with prostratin, drastically decreasing between nt positions 1000 and 3500,
324 but increasing again towards the 3' end between positions 6000 and 9000 (Fig. 6C, light
325 blue line). This is consistent with the role of pSer2 in relief of promoter-proximal pausing
326 (mediated by P-TEFb (65, 66)) and recruitment of splicing and RNA processing
327 machinery (mediated by CDK13/12 (66–69)). A significant reduction of RNAPII
328 occupancy (total, pSer5 and pSer2) was observed on the HIV genome when prostratin
329 reactivation was performed in the presence of SP (Fig. 6A-C, orange lines). This is
330 apparent at the HIV promoter, but most noticeable along the gene body. Almost no
331 pSer5 RNAPII is observed along the HIV genome beyond the promoter in SP treated
332 cells, while total and pSer2 RNAPII is still detected at the both the promoter and
333 towards the 3'end of the genome. Importantly, no significant differences were observed
334 in the occupancy of RNAPII (Total, pSer5, pSer2) on the promoter of GAPDH or in the
335 gene body of RPL13A (Fig. 6D-F).

336 In sum, these results indicate that long-term treatment of cells with 10 μ M SP
337 significantly reduce RNAPII occupancy on the HIV genome, and potently suppresses
338 RNAPII recruitment upon prostratin stimulation. These effects are not observed on the
339 promoter or ORF of housekeeping genes.

340

341 **Long-term effects of SP treatment on the cellular transcription apparatus and cell**
342 **cycle progression.**

343 Previous reports have suggested that short term treatment of cells with 10 μ M SP
344 selectively degrades the XPB subunit of TFIIH, leaving other components mostly
345 unaffected (46, 48). Additionally in short-term studies, using RNA-seq and uridine
346 analog 5-ethynyluridine (EU) incorporation assays Alekseev *et al* did not observe global
347 transcriptional defects in SP treated cells (51). We thus investigated the long-term
348 effects of SP treatment on components of the TFIIH complex, as well as on a few
349 important CDKs and respective cyclins. No significant differences were observed in the
350 mRNA expression of any of the TFIIH components in OM-10.1 cells treated with SP for
351 over 100 days (Fig. 7A, cells from 1D). However, at the protein level, although XPB was
352 by far the most downregulated protein (over 95%), a clear downregulation of other
353 components of TFIIH (namely XPD, p62, CDK7 and Cyclin H) was observed at all time
354 points, as compared to DMSO and EPL (Fig. 7B). In addition, in ACH-2 cells treated for
355 15 days with 10 μ M SP, a similar, but more modest reduction in XPD, Cyclin H and
356 CDK7 subunits of TFIIH was also observed (Fig. 7C). This downregulation of TFIIH
357 components was detected as early as 72 h after treatment of OM-10.1 cells with 10 μ M
358 SP and did not appear to magnify over time (Fig. 7D).

359 We also investigated whether long-term SP treatment affected the expression
360 and function of other critical CDKs and their Cyclin partners, such as P-TEFb (CDK9
361 and Cyclin T1) and cell cycle proteins CDK2, Cyclin E1 and Cyclin A. No significant
362 differences were observed between SP, vehicle or EPL treated OM-10.1 cells (Fig. 7B).

363 Recently, CDK7 was shown to be the primary kinase responsible for CDK9 Ser175
364 phosphorylation, thought to bias CDK9's binding from BRD4 to Tat (30). Additionally, it
365 was proposed that CDK7 phosphorylates CDK9 at Thr186, which is required for CDK9
366 activity, though CDK9 also autophosphorylates at this residue (30, 70). Given SP
367 reduction of CDK7 protein expression (Fig. 7B,D), we compared the levels of pThr186
368 and pSer175 CDK9 by Western blot in SP and vehicle treated OM-10.1 cells treated for
369 24 days (Fig. 7E). While potent depletion of XPB and approximately 60% reduction in
370 CDK7 was observed, the levels of total, pThr186 and pSer175 CDK9 remained
371 unchanged. Furthermore, by propidium iodide staining, no differences in cell cycle
372 progression between treatment groups were observed (Fig. 7F).

373 In sum, although long-term treatment of cells with 10 μ M SP results in a
374 reduction in protein levels of additional components of the TFIID complex besides XPB,
375 we did not observe differences in the expression of other important kinases, cyclins,
376 housekeeping genes, phosphorylation of CDK9 nor in cell cycle progression.

377

378 **Treatment of latently infected cells with SP reduces HIV transcription without** 379 **global dysregulation of cellular mRNA expression.**

380 Due to the critical nature of TFIID for RNAPII-mediated transcription, and the
381 significant loss of some of the components of TFIID, in addition to XPB (Fig. 3F, 6C, 7),
382 we investigated global cellular mRNA synthesis defects that may occur with SP
383 treatment. As such, we treated OM-10.1 cells for 15 days with either SP+ART or
384 DMSO+ART (once maximal HIV inhibition had been achieved in the SP treated cells),
385 followed by RNA extraction and mRNA-seq analysis. For this study, we included cells

386 stimulated or not with the PKC agonist, prostratin to assess effects on activation
387 dependent genes. HIV encoding genes were among the most significantly
388 downregulated genes in cells treated with SP compared to DMSO control, particularly
389 upon stimulation with prostratin (Fig. 8A, left and right panels, red points). Some of the
390 most significantly affected cellular genes are highlighted and labelled in blue [$p_{Adj} < 1e-10$
391 and $abs(\log_2\text{FoldChange}) > 1$ for SP vs DMSO comparisons (left and right panels,
392 Supplemental Tables S1-S2), and $p_{adj} < 1e-30$ with an $abs(\log_2\text{FoldChange}) > 2.5$ for
393 Stim vs No stim comparisons (middle two panels, Supplemental Tables S3-S4)]. In
394 unstimulated cells, treatment with SP resulted in the upregulation of 49 genes and
395 downregulation of 25 as compared to DMSO control (Fig. 8A left panel, $p_{Adj} \leq 0.05$,
396 $\log_2FC \geq abs(1)$). Prostratin stimulation led to the differential expression of 758 genes
397 in DMSO treated cells and 823 genes in SP treated cells (546 of which were
398 differentially expressed between conditions) (Fig. 8A, middle panels). SP treatment,
399 while specifically affecting the expression of some genes, does not appear to affect all
400 RNAPII-mediated transcription, in support of previous studies suggesting XPB depletion
401 by SP does not affect global cellular transcription (51). To determine more rigorously
402 what patterns of gene signaling pathways were affected by SP treatment, we performed
403 gene set enrichment analysis (GSEA) (71). A bubble-lattice plot was constructed to
404 highlight gene sets affected by SP treatment, where the color of the bubble represents
405 the normalized enrichment score (NES) (indicating the number and differential intensity
406 of the assessed genes in each indicated pathway) and statistical significance is
407 indicated by the size of the bubble (Fig. 8B). HIV encoded genes were significantly
408 down-regulated as compared to DMSO control, particularly under conditions of

409 prostratin stimulation, indicating the strong specific effect of SP treatment on HIV
410 transcription (Fig. 8B). Of note, with this type of bubble representation of the data, it is
411 important to keep in mind the size of the gene set, for instance the MYC targets have
412 modest downregulation with SP treatment, but since there are 200 genes in this set the
413 NES score is high. On the other hand, downregulation of HIV genes is greater, but
414 because there are lot fewer genes in this set the NES is smaller. Genes related to DNA
415 packaging (histone and chromatin-related genes) were significantly upregulated in both
416 unstimulated and prostratin stimulated SP treated cells (Fig. 8B). Conversely, MYC
417 targets, E2F targets, and Type I IFN receptor binding genes were downregulated with
418 SP treatment, which increased in significance upon prostratin stimulation (Fig. 8B).
419 Furthermore, cellular (endogenous) genes involved in the late phases of the HIV life
420 cycle (reactome gene sets (72)) were modestly downregulated upon stimulation, which
421 include genes involved in transcription, RNA processing and export from the nucleus
422 (Fig. 8B). IFN- γ response genes were slightly upregulated in unstimulated SP treated
423 cells, gaining significance upon stimulation (Fig. 8B). Other gene sets related to
424 transcription of the HIV genome, RNAPII transcription of snRNAs, RNAPII elongation,
425 IFN- α response, housekeeping and apoptosis were not substantially affected (Fig. 8B).
426 Heatmaps were constructed from transcripts per million (TPM) data to facilitate direct
427 comparison of gene expression with different lengths (Fig. 8C, Supplemental Table S5).
428 All HIV genes were clearly downregulated upon SP treatment and, although prostratin
429 stimulation increased HIV expression, this was substantially reduced compared to
430 DMSO treated cells (Fig. 8C, left panel). Because of the modest downregulation in
431 genes involved in the late phase of the HIV life cycle observed in the GSEA (Fig. 8B),

432 we examined the expression of individual genes within this set. Expression of
433 transcription related genes such as TFIIH, P-TEFb, the SEC, NELF, DSIF and RNAPII
434 genes do not appear to be substantially affected by SP treatment (Fig. 8D, left panel).
435 Furthermore, no obvious differences in expression of RNA splicing, export, capping or
436 cleavage genes between SP and DMSO treated cells (Fig. 8C middle panel). These
437 results are consistent with previous results, in which SP did not affect the transcriptional
438 machinery and cell cycle progression (Fig. 7), but blocked HIV reactivation (Fig. 4).
439 Nonetheless, SP treatment does curiously seem to upregulate a large subset of DNA
440 packaging complex genes (Fig. 8C, right panel), which is often associated with defects
441 in cell cycle progression (73). However, this upregulation might not be biologically
442 significant since, as mentioned above, no obvious differences in cell division were
443 observed in SP treated cells (Fig. 7).

444 In sum, SP treatment results in robust suppression of HIV transcripts both in
445 unstimulated or prostratin stimulated cells, consistent with the observations in Figs.1-5.
446 While the expression of a few specific gene sets were significantly impacted by SP
447 treatment, we hypothesize that their fold change may not be biologically significant
448 since we observe no major defects were observed in cell growth and viability.
449 Nevertheless, the effects of SP treatment at concentrations sufficient to potently
450 degrade SP will need to be assessed *in vivo*.

451

452 **DISCUSSION**

453 Inhibiting transcription from latent proviruses is an increasingly attractive approach for a
454 functional cure for HIV-1. The long-term transcriptional inhibition of HIV-1 may lead to a

455 sustained epigenetic repression with the potential to contain viral transcription even in
456 the absence of ART (43, 44). In addition, such transcriptional inhibition may reduce
457 “blips”, or moments of detectable viremia observed in some virally suppressed patients,
458 as well as chronic immune activation that stems from the continuing trickling of HIV
459 transcription and viral protein production observed under suppressive ART. To date,
460 there are unfortunately no FDA approved HIV-1 transcriptional inhibitors available. In
461 this study, we investigated the potential of the clinically approved drug SP, which targets
462 the host transcription factor XPB for proteasomal degradation, for the inhibition of latent
463 HIV-1 in various cell line models and primary CD4⁺ T cells from virally suppressed
464 individuals.

465 In agreement with previous studies, we observed a dose-dependent reduction of
466 XPB protein levels with SP treatment (Fig. 1B and 2B) (46–48, 51), which parallels a
467 dose-dependent reduction in HIV-1 replication (Fig. 1A and 2A). Here we showed that
468 specific shRNA knockdown of XPB resulted in similar HIV inhibition, suggesting that HIV
469 inhibition by SP treatment is specifically mediated by XPB protein levels (Fig. 3).
470 Importantly, EPL, a more specific MR antagonist, does not inhibit HIV nor degrades
471 XPB (Fig. 1C and 2C). Thus, HIV inhibition by SP is independent of MR antagonism and
472 dependent on the degradation of the host helicase XPB. Contrary to previous reports
473 that showed short term SP treatments selectively degrade XPB, leaving other subunits
474 of TFIIH relatively intact (47, 48, 51), we show that even after a short 72 h period the
475 levels of XPD, p62, CDK7 and Cyclin H, but not other cellular proteins, were
476 significantly reduced (Fig. 3F, 5C and 7). These effects seem to be somewhat cell type
477 specific, since they were more pronounced in OM-10.1 as compared to ACH-2 cells

478 (Fig. 7B vs 7C). This could be explained either by more modest XPB degradation in
479 ACH-2 cells (Fig. 1B vs 2B) or it could be related to the absence of a functional Tat-TAR
480 feedback loop in ACH-2 cells and this warrants further investigation. It remains to be
481 determined whether loss of other TFIIH subunits is due to destabilization of the complex
482 or to off-target proteasomal degradation. Of note, shRNA knockdown of the XPB gene,
483 *ERCC3*, does not result in the same degree of TFIIH components loss (Fig. 3F). While
484 reduction of XPB with *ERCC3* shRNA knockdown was around that observed with 1.8
485 μ M SP treatment, we do not observe any loss of XPD, and a more modest loss of CDK7
486 and Cyclin H. Importantly, *ERCC3* knockdown showed no cytotoxic or cytostatic effects
487 (Fig. 5C). Although shRNA knockdown of XPB resulted in an ~80% reduction of XPB
488 protein (similar to treatment with 1.8 μ M SP, Fig. 3A), with less effects on other TFIIH
489 components, it reduced HIV mRNA levels to a greater degree than 1.8 μ M SP
490 treatment, further suggesting that development of more specific XPB degraders may
491 improve on SP potency and could potentially avoid unwanted off-target transcriptional
492 activity as well as the other known effects of SP on the MR.

493

494 Treatment of latently infected cells with SP results in a rapid, robust suppression of HIV
495 transcription, and reactivation using LRAs is significantly inhibited (Fig. 4). Importantly,
496 SP treatment blocks the strong HIV-1 reactivation by PMA/ionomycin in CD4⁺ T cells
497 explanted from HIV-infected virally suppressed individuals (Fig. 4E). While levels of HIV
498 reactivation were substantially reduced with SP treatment (average of 27.6-fold), the
499 levels of the housekeeping gene, *POLR2A* (encoding a subunit of RNAPII), were
500 modestly elevated (average of 2.5-fold) (Fig.4F). The slight increase in *POLR2A*

501 observed in primary cells upon SP treatment requires further investigation to determine
502 whether this is a direct consequence of SP activity or is related to inhibition of HIV
503 transcription. Of note, even if not statistically significant ($p=0.78$), the mRNA-seq
504 analysis in OM-10.1 cells showed a slight increase in *POLR2A* expression in cells
505 treated with SP stimulated with prostratin, as compared to DMSO (log2FC of 0.5).
506 Furthermore, there were no obvious changes in expression of other RNAPII subunits
507 (Fig. 8D, middle panel).

508 Importantly, SP treatment was shown to be sufficient to maintain HIV p24
509 production below the limit of detection in the absence of ART (Fig. 5A). These results
510 highlight the potential of using SP as a single drug to maintain HIV-1 suppression once
511 deep latency has been established, in the absence of ART. However, regardless of
512 treatment duration in the latent models tested, transcription remains detectable and
513 once SP treatment is interrupted there is rapid transcriptional rebound to control levels
514 (Fig. 5B). This was not observed with treatment with dCA, where longer treatment
515 periods led to undetectable levels of viral RNA and sustained viral inhibition when
516 treatment was interrupted (41, 43). The reasons for this difference may be two-fold, on
517 one hand since a host component of a transcription factor complex is involved, as soon
518 as XPB is produced again above a certain threshold it can participate and jumpstart
519 transcription from the viral promoter. In fact, the levels of XPB protein become elevated,
520 and remain so for at least 9 days, upon removal of SP (Fig.5C), possibly due to
521 elevated XPB mRNA levels with SP treatment (Fig. 1F). On the other hand, since
522 transcriptional inhibition is not fully complete, the residual level of transcription may
523 preclude epigenetic repressive mark deposition for complete silencing of the promoter

524 locus. Future studies will address the benefits of combining SP treatment with other
525 transcriptional inhibitors, such as dCA.

526

527 The RNAPII CTD tail contains 52 repeats of the heptad $Y_1S_2P_3T_4S_5P_6S_7$ that serves as
528 a hotspot for post-translational modification to fine-tune transcription (66, 74–76).

529 Hypophosphorylated RNAPII is recruited to the promoter and is primed for activation by

530 phosphorylation of Ser7 by the CDK7 subunit of TFIIF. Additional phosphorylation of

531 Ser5 by CDK7 activates RNAPII to initiate transcription and escape the promoter, as

532 such pSer5 levels are highest at the TSS but decrease toward the end of genes.

533 Promoter-proximal pausing of RNAPII occurs due to repressive factors (DSIF and

534 NELF) and occlusion by Nuc-1. RNAPII pausing is relieved through CDK9

535 phosphorylation DSIF, NELF and Ser2 of RNAPII CTD, resulting in a pSer2 peak just

536 downstream of the TSS. Further phosphorylation of Ser2 occurs as RNAPII moves

537 along the gene body, mediated by CDK12/13, resulting in increasing pSer2 levels

538 towards the 3' end of genes in the gene body and is associated with recruitment of RNA

539 processing and splicing factors (67, 68, 77, 78). Spironolactone treatment results in a

540 loss of RNAPII (total, pSer5 and pSer2) occupancy throughout the HIV genome,

541 particularly noticeable upon LRA stimulation (Fig. 6A-C). In unstimulated cells, little

542 RNAPII is recruited to the promoter of either SP or DMSO treated cells, given the low

543 basal HIV expression in these cells. Upon reactivation with prostratin, some RNAPII

544 was still detected at the TSS in SP treated cells, even with the robust loss of XPB. This

545 is not unexpected, since RNAPII is brought to the promoter during PIC formation, prior

546 to TFIIF recruitment (14, 15, 17). However, since HIV transcription depends on the

547 positive feedback loop driven by Tat, a reduction in elongation would result in a loss in
548 recycling of RNAPII and reduced overall RNAPII occupancy at the promoter. RNAPII
549 was barely observed in SP+ART treated cells downstream from the promoter,
550 compared to robust recruitment in DMSO+ART treated cells upon transcriptional
551 reactivation. This is consistent with the role of TFIIH in initiation and promoter clearance
552 (16, 18, 19). Significantly, changes in RNAPII occupancy (total, pSer5 and pSer2 CTD)
553 were not observed at either the promoter of GAPDH or the ORF of RPL13A (Fig. 6D-F),
554 suggesting that the HIV-1 genome is especially sensitive to perturbation of TFIIH and
555 the reduction in RNAPII recruitment is specific to the HIV-1 genome. This is consistent
556 with our own results (Fig. 8) and previous work which revealed genes highly dependent
557 on NF- κ B experience reduced RNAPII recruitment with SP treatment, while other more
558 constitutively expressed genes were not affected (47). NF- κ B has been shown to recruit
559 TFIIH to the inactive RNAPII poised at the HIV TSS, to allow CDK7 phosphorylation of
560 RNAPII CTD (53).

561

562 TFIIH is a critical general transcription factor, involved in the initiation of transcription
563 most RNAPII-dependent genes, including HIV (14, 15, 19, 53, 79). However, XPB has
564 been proposed to be dispensable for RNAPII-mediated transcription (51). Despite SP
565 treatment mediating substantial XPB degradation and moderate loss of other TFIIH
566 components, SP seems well tolerated by cells treated long-term. In addition, SP does
567 not appear to interfere with cell cycle progression, it does not cause significant losses of
568 P-TEFb or cell cycle proteins, and has no effect on phosphorylation of CDK9 (Fig. 1G,
569 2G, 3, 7). In agreement, our mRNA-seq analysis suggests SP treatment specifically and

570 potently inhibits HIV expression, while more modestly affecting a subset of cellular
571 genes (Fig. 8). We cannot exclude the possibility that HIV transcription is inhibited
572 indirectly through SP-mediated effects on cellular proteins, since GSEA revealed
573 modest reduction in genes involved in the late phase of the HIV life cycle (Fig. 8B).
574 However, SP did not cause obvious changes in the mRNA expression (TPM count data)
575 of RNA processing genes (capping, cleavage, splicing or export) or RNAPII-related
576 transcription factors (TFIIH, P-TEFb, super-elongation complex, NELF or DSIF) that
577 could have potentially explained the potent downregulation of HIV mRNA expression
578 (Fig. 8C). Intriguingly, histone and chromatin related genes (DNA packaging) were
579 among the most upregulated genes upon SP treatment (Fig. 8B-C). However, these
580 changes are probably not significant, since no changes in cell cycle progression were
581 observed (Fig. 7).

582 Despite a ~95% reduction in XPB levels, and between 20 and 60% reduction in
583 some other components of TFIIH with 10 μ M SP treatment (Fig. 7), we hypothesize
584 there is sufficient amounts of this complex to support basal transcription. Activation
585 dependent genes, such as cytokines and HIV, which require recruitment of large
586 amounts of TFIIH may be more susceptible to XPB loss (47). These results are not
587 unexpected and are consistent with previous reports showing SP treatment does not
588 globally reduce transcription, but rather more selectively inhibits HIV and NF- κ B-
589 dependent transcription (47, 48, 51). Interestingly, mRNA-seq differential expression
590 analysis revealed significant downregulation of *MYC* and *MYC*-target genes (Fig. 8B).
591 This is consistent with the known role of XPB regulating c-*MYC* expression through
592 FUSE binding protein (FBP) and the FBP Interacting Repressor (FIR) which bind to the

593 Far Upstream Sequence Element (FUSE) (80, 81). FBP/FIR act in response to cellular
594 signals to control RNAPII pausing and release through interactions with the XPB subunit
595 of TFIIH to regulate *MYC* expression (82, 83). It is proposed that FUSE and FIR binding
596 to TFIIH regulates activation-dependent, but not basal *MYC* expression (82). The level
597 of TFIIH in cells is tightly regulated and correlates with transcriptional activity (84). Thus,
598 we expect that inducible genes that become transcriptionally very active upon
599 stimulation may be more susceptible to XPB degradation than those transcribed at a far
600 lower rate.

601 Further studies are needed to determine the full extent by which SP treatment
602 inhibits HIV transcription, directly via XPB degradation or indirectly via other altered
603 cellular genes, and the mechanisms behind the increased susceptibility of HIV to SP
604 treatment, compared to the majority of cellular genes (51).

605

606 Although SP has been very useful tool to tease out the role of XPB in transcription, it will
607 take some additional monitoring for it to be explored for block-and-lock approaches in
608 the clinic. The concentrations of SP needed for maximal XPB degradation and HIV-1
609 inhibition (10 μM) is well above the peak serum concentrations reached in healthy
610 volunteers administered 100 mg SP daily for 15 days of around 0.19 μM . The area
611 under the curve (AUC) at day 15, reflecting the actual body exposure to the drug was
612 0.55 μM , which would result in suboptimal XPB degradation (Fig.1B and 2B) (85).
613 However, SP is used to treat women experiencing hair loss at 200 mg daily without
614 negative effects and transgender women take up to 400 mg daily pre-surgery (86, 87).
615 Additionally, the FDA has approved a four-week treatment of 400 mg daily SP to

616 diagnose hyperaldosteronism (88). The most common side effects reported at high
617 doses of SP are gynecomastia, and the more serious hyperkalemia, which requires
618 monitoring (89). XPB is also involved in nucleotide excision repair and has been
619 proposed as a means to sensitize cells to platinum-based cancer therapies (46). The
620 FDA noted that rats treated with 500 mg/kg/day (about 25 times the human
621 recommended daily dose of 200 mg per day) had an increase in occurrence of benign
622 tumors. The risk of treating individuals with a dose of SP high enough to degrade XPB
623 would need to be assessed, and whether or not these side effects will be a major
624 drawback for clinical testing remains to be seen. SP has, however, great potential to be
625 used in combination with other therapies, given its very quick effects on HIV. One could
626 foresee the initial use of high doses of SP to reduce HIV-1 transcription to or below the
627 level of detection, at which point SP doses could be reduced and another inhibitor, such
628 as a Tat inhibitor, could be introduced to bring about a state of “deep latency”, from
629 which rebound would be very difficult to occur. Future studies will investigate this
630 combination therapy. Furthermore, combination treatments may allow the use of
631 reduced dosages.

632 Altogether we showed here that targeting a host component of a transcription
633 factor complex, XPB, reduces HIV-1 transcription and reactivation from latency without
634 obvious adverse effects on cellular transcription. We show that SP acts additively with
635 ART to reduce HIV-1 transcription and block reactivation from latency upon stimulation
636 with LRAs. Even given some concerns with the high doses of SP required for XPB
637 degradation *in vivo*, our results warrant the exploration of SP as a therapeutic in “block-
638 and-block” approaches for a functional cure.

639

640 **MATERIALS AND METHODS**

641 **Cell lines and cell culture.** OM-10.1 and ACH-2 cells were obtained through the NIH
642 AIDS Reagent Program (from Dr. Butera, # 1319 and Dr. Folks, # 349 respectively).
643 Cells were maintained in RPMI 1640 (Gibco) supplemented with 2.0 mM L-
644 glutamine/100 U/ml penicillin/100 µg/ml streptomycin (Gibco) and 10% heat-inactivated
645 FBS (Atlas Biologicals, catalog no. FS-0500-AD) (complete RPMI) and cultured at 37°C
646 and 5% CO₂. Cells were passaged every three days and media was replaced with the
647 indicated concentrations of SP, EPL or DMSO and an ART cocktail (200 nM Lamivudine
648 (AIDS Reagent), 200 nM raltegravir (Selleckchem, MK-0518), 100 nM efavirenz (AIDS
649 Reagent)). SP was purchased from Sigma-Aldrich (# 1619006), dissolved in DMSO
650 (Fisher Scientific, # BP231-100). EPL was purchased from Sigma-Aldrich (# E6657-
651 10MG) and dissolved in DMSO. Cell concentration and viability was measured using the
652 TC20 automated cell counter (Bio-Rad, # 1450102) and trypan blue staining.

653

654 **Infection of Hut78 cells with SIVmac239.** Hut78 cells were infected as previously
655 described (90). Briefly, cells were infected overnight with SIVmac239. Cells were then
656 washed with phosphate buffer saline, split into 3 flasks and treated with DMSO, EPL or
657 SP (10 µM) for 12 days. SIV replication was assessed every 3 days post-infection, by
658 measuring the viral capsid in the supernatant with p27 ELISA (Advances Bioscience
659 Laboratories, # 5436).

660

661 **Determination of SP half-life in cell culture.** Stability of SP was evaluated in cell
662 culture using an ABSciex 5500 mass spectrometer run in multiple reaction monitoring
663 mode. Cells were treated with 10 μ M SP (with or without ART) and the concentration of
664 SP was evaluated before and after 72 h incubation, at multiple time points. Cells were
665 resuspended and treated with 2-times v:v acetonitrile to lyse the cells, precipitate
666 cellular protein, and inactivate the virus. Samples were filtered through a 0.2 micron
667 filter plate prior to injection onto the LC-MS/MS. LC conditions used a Thermo Betasil
668 C18 5 μ , 50x2.1mm column with a linear gradient of A: water + 0.1% formic acid and B:
669 acetonitrile with 0.1% formic acid (10%B from 0-5 min, 90%B at 2-3 min, 10%B at 4-6
670 min). Detection of SP used the mass transition of 417.3 \rightarrow 341. Half-life was calculated
671 by plotting the natural log of the percent remaining compound verses time where $T_{1/2} =$
672 $\ln(2)/\text{slope}$ of the linear regression.

673

674 **p24 ELISA.** The amount of HIV p24 capsid protein produced in the supernatant of
675 cultured cells was quantified using the antigen capture assay kit from Advanced
676 BioScience Laboratories, Inc. (# 5447), and performed according to manufacturer's
677 protocol.

678

679 **MTT Cell Proliferation assay.** The viability and proliferation of cells treated with
680 increasing concentrations of SP and EPL were measured using the MTT assay
681 according to the manufacturer's protocol (ATCC, # 30-1010K). Cells were cultured in 96-
682 well plates (in triplicate per drug concentration) for 72 h prior to addition of the MTT
683 reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide).

684

685 **Western blots for OM-10.1, ACH-2 and HUT-78 cells.** Cells were collected, washed
686 and lysed with a 20 mM HEPES [pH 8.0], 100 mM KCl, 0.2 mM EDTA, 5 mM β -
687 mercaptoethanol, 0.1% IGEPAL CA-630, 10% glycerol, and supplemented with
688 complete EDTA-free protease inhibitor cocktail (Roche, # 4693132001) on ice for 10
689 min. For pSer175 and pThr186 CDK9 Western blots we added phosphatase inhibitor
690 cocktail 3 (Sigma-Aldrich, # P0044-1ML). Protein concentration was determined using
691 the Bio-Rad Protein Assay Dye (# 50000006). Equal amounts of total protein extract
692 were loaded, alongside the Precision Plus ProteinTM All Blue protein standards (Bio-
693 Rad, # 1610373) onto a pre-cast 4 – 20% gradient stain-free protein gel (Bio-Rad, #
694 4568094). Proteins were then transferred to a nitrocellulose membrane using the Bio-
695 Rad Trans-Blot® TurboTM system (# 1704270). Membranes were blocked with 5% milk
696 and probed with the following antibodies: anti-XPB (Sigma-Aldrich, # X0879, diluted
697 1:2000), anti-XPB (Cell Signaling Technology, # 11963S, diluted 1:1000), anti-CDK7
698 (Santa Cruz, sc-7344, diluted 1:500), Cyclin H (Cell Signaling Technology, # 2927S,
699 diluted 1:1000), p62 (Santa Cruz, sc-25329, diluted 1:500), Cyclin A2 (Cell Signaling
700 Technology, # 4656S, diluted 1:1000), Cyclin E1 (Cell Signaling Technology, # 4129S,
701 diluted 1:500), CDK2 (Cell Signaling Technology, # 25465S, diluted 1:1000), CDK9
702 (Cell Signaling Technology, # 2316S, diluted 1:1000), pThr186-CDK9 (Cell Signaling
703 Technology, # 2549S), pSer175-CDK9 (kind gift from Jonathan Karn), β -tubulin
704 (proteintech, # 66240-1-IG, diluted 1:10,000) and GAPDH (Santa Cruz, sc-32233,
705 diluted 1:1000) in 3% BSA (or 5% milk for the β -tubulin antibody). Secondary antibodies
706 were anti-mouse (GE Healthcare, # NA931-1ML) diluted 1:10,000 or anti-rabbit (Sigma-

707 Aldrich, catalog no. A0545), diluted 1:15,000, both conjugated to horseradish
708 peroxidase. Bands were visualized using the Amersham ECL Prime Western Blotting
709 Detection Reagent (GE Healthcare, # RPN2236) and imaged on the Bio-Rad ChemiDoc
710 XRS+ System.

711

712 **Quantitative PCR of cell-associated RNA.** Total RNA was extracted from cells using
713 RNeasy kit (Qiagen, # 74106). RNA was DNase treated using the TURBO DNA-free™
714 kit (Invitrogen, # AM1907). cDNA was synthesized using random hexamer primers and
715 SuperScript III First Strand Synthesis kit (Invitrogen, # 18080051). qPCR was performed
716 using SensiFAST™ SYBR ® No-ROX Kit (Bioline, # BIO-98020). Samples were run in
717 duplicate or triplicate. The mRNA expression was normalized to RPL13A mRNA
718 expression and the relative abundance was calculated ($\Delta\Delta Ct$).

719

720 **qPCR primers**

721 RPL13A primers were purchased from Bio-Rad, PrimePCR SYBR Green Assay:

722 RPL13A Human #10025637. XPB-F: GGATGAGTCAGGCACCAAAGT, XPB-R:

723 CTGGAGAGAAGGCTTCCAAGAA, XPD-F: CGACTACATCTACCCCGAGCA, XPD-R:

724 CATGATCAGGGCCAACAGGGA, CCNH-F: TCACCCAGGATAATAATGCTCA,

725 CCNH-R: CAGTATCTGTTCAAGTGCCTTCT, CDK7-F:

726 GGAGCCCCAATAGAGCTTATACA, CDK7-R: TCCACACCTACACCATACATCC,

727 GTF2H1-F GACCTTGTTGTGAGTCAAGTGA, GTF2H1-R:

728 CCTGCTTATGATTGGATGTGGAA, GTF2H2-F: CGTATGGGATTCCTCAGCAC,

729 GTF2H2-R: AGCCTCCTAATGTAAGCCCTG, GTF2H3-F:

730 GAATGGCAGACTTGGAGACTTC, GTF2H3-R: GCAAAGTTTCTGTATGTTGACCC,
731 GTF2H4-F: ACCCCATTTTCCGCCAGAAC, GTF2H4-R:
732 CGGCGTACTTGTCAAGGGAG, GTF2H5-F: AAGACATTGATGACACTCACGTC,
733 GTF2H5-R: GGGAAAAAGCATTTTGGTCCATT, MNAT1-F:
734 GGTTGCCCTCGGTGTAAGAC, MNAT1-R: AGTTGCTCTTTCTGAGTGGAGT,
735 AllHIVmRNA-F: TTGCTCAATGCCACAGCCAT, AllHIVmRNA-R:
736 TTTGACCACTTGCCACCCAT, HIV early *gag*-F: GCGACTGGTGAGTACGCCAA, HIV
737 early *gag*-R: CCCCTGGCCTTAACCGAATTT.

738

739 **ChIP qPCR primers**

740 123-F: CCCTGATTGGCAGAACTACACAC, 123-R:
741 TCTACCTTATCTGGCTCAACTGGT, 326-F: GACAGCCGCCTAGCATTTCAT, 326-R:
742 CCACGCCTCCCTGGAAAGT, 412-F: CGAGAGCTGCATCCGGAGTACT, 412-R:
743 GAGGCTTAAGCAGTGGGTTCC, 1097-F: GCAGTCCTCTATTGTGTGCATCAA, 1097-
744 R: CCTGTGTCAGCTGCTGCTTG, 1477-F: CCATCAATGAGGAAGCTGCAGAA, 1477-
745 R: GGTGGATTATGTGTCATCCATCCT, 3582-F: CAGAAATACAGAAGCAGGGGCAA,
746 3582-R: GTGTGGGCACCCTTCATTCTT, 6352-F: GAGCAGAAGACAGTGGCAATGA,
747 6352-R: CACAGGTACCCATAATAGACTGTG, 8885-F:
748 CCTGCTGTAAGGGAAAGAATGAGAC, 8885-R: CACCTCTTCCTCCTCTTGTGCT,
749 GAPDH-F: CCTCACGTATTCCCCAGGTTTA, GAPDH-R:
750 AGCCACACCATCCTAGTTGCCT.

751

752 **Cell cycle stage analysis.** OM-10.1 cells were treated long-term (80 to 100 days) with
753 ART and 10 μ M SP, 10 μ M EPL or DMSO, being split and treated every 72 h. After 72 h
754 of incubation, 1 $\times 10^6$ cells were collected per condition (in triplicate) for cell cycle
755 analysis. All centrifugation steps were performed at 500 x g for 5 min. Fixation: Cells
756 were washed in 1X DPBS (Gibco, # 14190-250) and then fixed in 66% ice cold ethanol,
757 on ice, for at least 2 h or stored at 4 °C until the next day. Staining: Fixed cells were
758 equilibrated to room temperature, resuspended and pelleted. Fixing solution was
759 aspirated and cells were washed with 1X DPBS. The pellet was resuspended in 0.5 ml
760 of FxCycle PI/RNase staining solution (Invitrogen, # F10797) and stained for 15-30 min
761 at RT, protected from light. Cells were acquired on a BD LSRII flow cytometer using the
762 561nm laser with the 617/25 band pass filter.

763

764 **Transduction of OM-10.1 cells.** The Mission shRNAs targeting ERCC3 and GFP in the
765 pLKO.1-puro plasmid were purchased from Sigma-Aldrich (SHCLNG:
766 TRCN0000359264 and SHC204 respectively). First, lentiviral vectors were prepared by
767 transient transfection of 293T cells, with the shRNA vector and packaging plasmids
768 pMD2.g and psPAX2 (gifts from Didier Trono, Addgene plasmid # 12259 and # 12260)
769 at a molar ratio of 2:1:1 respectively. Transfections were performed using TransIT-LT1
770 transfection reagent (Mirus Bio LLC, # MIR 2305) as per manufacturer's instructions.
771 Lentiviral virus-like particles (VLPs) were collected and filtered through a 0.45 μ m filter,
772 to remove cellular debris, at 48 and 72 h post-transfection. Filtered VLPs were
773 combined, quantified and stored at -80°C until use. Three days prior to transduction,
774 media was changed on OM-10.1 cells to remove ART, to allow successful transduction

775 with the VLPs. OM-10.1 cells were transduced with equal M.O.I.s of lentiviral vectors in
776 T25 flasks as follows: pLKO.1-shGFP, pLKO.1-ERCC3, pLKO.1-shGFP + 1.8 μ M SP,
777 and pLKO.1-shGFP + 6.3 μ M SP, in the presence of the indicated concentrations of SP.
778 VLPs were added to cells and a non-transduced control was included. Cells were
779 washed after an overnight incubation and ART (+/- the indicated SP concentrations)
780 was added back to the media. Two days later, cells were selected with 1.5 μ g/ml
781 puromycin (Gemini Bio-Products, # 400-128P). Cells were maintained in puromycin
782 supplemented media treated with the indicated concentrations of SP every 3 days. Ten
783 days post-puromycin selection cells were harvested for XPB protein analysis by
784 Western blot and RNA expression analysis of *ERCC3* and HIV by RT-qPCR.
785 Additionally, supernatant was collected from each transduction for quantification of HIV
786 capsid protein by p24 ELISA. Cells were counted and viability was determined by trypan
787 blue staining, using the TC20 automated cell counter.

788

789 **Latent HIV-1 reactivation.** OM-10.1 cells (after 45 days of treatment with ART and 10
790 μ M SP or DMSO) and ACH-2 cells (after 15 days of treatment with ART and 10 μ M SP
791 or DMSO) were stimulated (or not) with 1 μ M prostratin (Lc Laboratories, # P-4462-
792 1MG), 10 ng/ml TNF- α (Biolegend, # 570104), 1 μ M SAHA (Lc Laboratories, # V-
793 8477_1g) or 20 nM PMA (Sigma-Aldrich, # P1585-1MG). This was done in the presence
794 of ART and either 10 μ M SP or DMSO. After 8 h, p24 production was measured in the
795 supernatant by ELISA and cells were collected for RNA extraction and RT-qPCR
796 analysis.

797 J-Lat A1 cells were treated with 160 nM PMA or 1 ng/ml TNF- α for 2 h and then
798 treated overnight with 10 μ M SP, EPL or DMSO, after which the mean fluorescence
799 intensity of GFP was measured by flow cytometry. J-Lat 11.1 cells were treated with 8
800 nM PMA for 2h following overnight treatment with 10 μ M SP, EPL or DMSO.

801

802 **Isolation, expansion and reactivation of primary CD4+ T cells**

803 Resting CD4+ T cells were isolated from 7 ART-treated, virally suppressed, HIV-1-
804 infected individuals through magnetic negative depletion as previously described (91)
805 and treated with SP (10 μ M) or mock (DMSO) for 24 hrs. The fusion inhibitor enfurvitide
806 was supplemented to the cell culture to prevent new rounds of infection. In the final 6
807 hrs of culture, the cells were treated with PMA (50 ng/mL) and ionomycin (1 μ M) to
808 induce maximum *ex vivo* latency reversal as previously described (64). Cell-associated
809 HIV-1 RNA was extracted using TRIzol phase separation and DNase treatment. HIV-1
810 RNA expression was measured by RT-qPCR as cell-associated polyadenylated HIV-1
811 levels per million CD4+ T cells (92, 93). Cellular gene expression (*POLR2A*, Thermo
812 Fisher, # 4331182) was measured as cell-associated polyadenylated *POLR2A* levels
813 per million CD4+ T cells.

814

815 **RNA polymerase II ChIP.** Three independent treatments of OM-10.1 cells with ART
816 and 10 μ M SP or DMSO for ~70 days were stimulated (or not) with 1 μ M prostratin for 8
817 h. Supernatant and cells were collected for p24 ELISA and RT-qPCR analysis
818 respectively and remaining cells were crosslinked with 1% formaldehyde for 10 minutes
819 and quenched by 0.125 M glycine for 5 min at room temperature. Cells were lysed using

820 an SDS lysis buffer and lysates were sonicated 16 times for 10 s bursts on ice to
821 generate sheared chromatin of 200 to 400 nucleotides, in the presence of complete
822 EDTA-free protease inhibitor cocktail (Roche, # 4693132001) and phosphatase inhibitor
823 cocktail 3 (Sigma-Aldrich, # P0044-1ML). The protein concentration of the precleared
824 sonicated lysate was determined using the Bradford protein assay. A 4% fraction of
825 each sonicated lysate was reserved for the input control. 4 μ g (per condition) of each of
826 the following antibodies, total RNAP II (Millipore, # 05-623), pSer5 RNAPII CTD (Active
827 Motif, # 61986), pSer2 RNAPII CTD (Active Motif, # 61984) or isotype controls, mouse
828 IgG (Invitrogen, # 31903) or rat-IgG (Thermo Fisher Scientific, # 029602) were
829 conjugated to Protein G Dynabeads (Thermo Fisher Scientific, # 10003D) for 20 min,
830 rotating at RT. A total of 500 μ g pre-cleared protein lysate was added to the conjugated
831 beads for IP overnight with rotation at 4° C. Dynabeads were washed, using magnetic
832 separation. Samples were then treated (including input samples) with freshly prepared
833 digestion buffer (10 mM Tris-HCl, 1 mM EDTA, 0.5% SDS, 10 μ g/ml RNase A (Thermo
834 Scientific, # EN0531)) at 37°C for 30 min. Cross-links were reversed for at least 4 h
835 shaking (1000 rpm) at 65°C with 200 nM NaCl. DNA samples were then treated with
836 proteinase K (Fisher Scientific, # BP1700100) shaking (1000 rpm) at 60°C for 1 h.
837 Dynabeads were removed and DNA was purified using the QIAquick PCR Purification
838 kit (QIAGEN, # 28106). qPCR was performed in 384-well format using SensiFASTTM
839 SYBR® No-ROX Kit (Bioline, # BIO-98020). The average CT value of the IgG only
840 background for each primer was subtracted from the CT value using the HIV-1 primer.
841 The data were then normalized to input values using the following formula:
842 % of input = $[2^{-\Delta\text{CT}}(\text{IP}) - 2^{-\Delta\text{CT}}(\text{IgG})] \times 4$.

843

844 **mRNA-seq library preparation**

845 Total RNA was extracted using the RNeasy kit (Qiagen, # 74106). RNA was DNase
846 treated using the TURBO DNA-free™ kit (Invitrogen, # AM1907). Total RNA quantified
847 on the Qubit 2.0 fluorometer and run on the Agilent 2100 Bioanalyzer RNA pico chip
848 (Agilent Technologies, Santa Clara, CA) for quality assessment. mRNA was selectively
849 isolated from total RNA (500ng) using poly-T oligos attached to magnetic beads
850 according to the manufacturer's guidelines in the NEBNext poly(A) mRNA magnetic
851 isolation module (Cat. #: E7490, NEB, Ipswich, MA). The library preparation from the
852 enriched mRNA was conducted according to NEBNext Ultra II Directional RNA kit (Cat.
853 # E7760, NEB, Ipswich, MA). Briefly, the enriched mRNA samples were chemically
854 fragmented at 94°C for 15 minutes. The fragmented RNA was random hexamer primed
855 and reverse transcribed to generate the first strand cDNA. The second strand was
856 synthesized after removing the RNA template and incorporating dUTP in place of dTTP.
857 The incorporation of dUTP quenches the second strand during the PCR amplification
858 step later and therefore the strand information was preserved. The purified ds cDNA
859 was end repaired and adenylated at their 3' ends. A corresponding 'T' nucleotide on the
860 adaptors was utilized for ligating the adaptor sequences to the ds cDNA. The adaptor
861 ligated DNA was purified and PCR amplified to incorporate unique barcodes to generate
862 the final libraries. The final libraries were validated on the bioanalyzer DNA chips,
863 pooled equally and loaded onto the NextSeq 500 for sequencing with 2 x 80bp paired-
864 end chemistry.

865

866 **Read mapping and differential expression analysis**

867 Raw FASTQC files were filtered for read quality using trim-galore version 0.6.1 with the
868 following options--clip_R1 5 --clip_R2 5 --trim-n --paired
869 (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Trimmed reads were
870 aligned to the human transcriptome (GRCh38v100) with the HIV genome added as an
871 additional chromosome (REFSEQ release 202). To align transcripts, salmon version
872 v0.14.1 was used with the --validateMappings option (94). Aligned transcriptomes were
873 mapped to genes and differential gene expression was called using tximportv1.16.1 and
874 DESeq2v1.28.1 respectively (95, 96). TPMs were determined using the
875 countsFromAbundance = "lengthScaledTPM" option in tximport. All plots were
876 generated in R. GSEAs were computed from the GSEA app v4.1.0 using TPMs as
877 counts and publicly available gene sets current as of October 2020 (71). DESeq2 and
878 TPM data can be found in Supplemental Tables S1-S5.

879

880 **Statistical analysis.** Statistics were performed using GraphPad Prism, and a P value of
881 <0.05 was considered significant for all comparisons. *P < 0.05, **P < 0.01, ***P <
882 0.001. ****P<0.0001. NS unless indicated. Data is presented as the mean of the
883 indicated replicates with error bars indicating the SD. The two-tailed T-test was used
884 where applicable, and a paired T-test was used for reactivation studies to compare
885 stimulated to unstimulated conditions. Comparison of multiple conditions were
886 performed using a two-way analysis ANOVA repeated-measures test (Dunnett or Holm-
887 Sidak).

888

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1177

1178 **FIGURE LEGENDS**

1179 **Figure 1. SP treatment inhibits latent HIV-1 transcription in the OM-10.1 cell line**
1180 **model of latency.**

1181 (A) OM-10.1 cells were treated with ART and the indicated concentrations of SP for 72
1182 h, after which the amount of HIV capsid was measured in the supernatant by p24
1183 ELISA. The IC₅₀ value was calculated using GraphPad Prism. The line represents the
1184 mean of three independent experiments, each with three technical replicates. Error bars
1185 represent SD.

1186 (B) Cells treated in (A) were collected for Western blot analysis with the indicated
1187 antibodies. Protein abundance was calculated by normalization to GAPDH levels and
1188 plotted relative to the DMSO control. A representative blot is shown above the
1189 quantification of three independent experiments. Error bars represent SD.

1190 (C) OM-10.1 cells were treated with ART and the indicated concentrations of EPL for 72
1191 h and the IC₅₀ values calculated as in (A).

1192 (D) Cytotoxicity of SP (red) and EPL (blue) treated cells in OM-10.1 cells treated for 72
1193 h with increasing concentrations of SP was measured using an MTT assay and plotted
1194 as a percentage of the DMSO control. Error bars represent SD from three independent
1195 experiments.

1196 (E) OM-10.1 cells were split and treated every 72 h in the presence of ART with 10 μM
1197 SP (red), EPL (light blue) or vehicle only (DMSO, dark blue). HIV capsid production was
1198 quantified by p24 ELISA. The dotted black line at 3 pg/ml represents the limit of
1199 detection (l.o.d). This plot is representative of three independent experiments.

1200 (F) Cell associated HIV mRNA levels (red) and *ERCC3* (XPB) mRNA levels (dark red)
1201 were measured at multiple time points during the long-term treatments of OM-10.1 with
1202 10 μ M SP in (D). cDNA was prepared from RNA extracted from cells pellets and qPCR
1203 performed with primers in the Nef region. The results were normalized as the number of
1204 viral mRNA copies per RPL13A mRNA and plotted relative to the DMSO treated control
1205 (blue, set to 100% for each time point). Error bars represent SD from the qPCR.

1206 (G) The number of viable cells per ml of culture (left panel) and percentage viable cells
1207 (right panel), respectively, were measured by trypan blue staining and an automated
1208 cell counter every 72 h during the long-term treatments of OM-10.1 cells with 10 μ M SP,
1209 10 μ M EPL or DMSO only.

1210

1211 **Figure 2: Tat-TAR independent inhibition of the ACH-2 T cell model of HIV latency**
1212 **and suppression of SIV infection with SP treatment.**

1213 (A) ACH-2 cells were treated with ART and the indicated concentrations of SP for 72 h,
1214 after which the amount of HIV capsid was measured in the supernatant by p24 ELISA.
1215 The IC50 value was calculated using GraphPad Prism. The line represents the mean of
1216 three independent experiments, each with three technical replicates. Error bars
1217 represent SD.

1218 (B) Cells treated in (A) were collected for Western blot analysis with the indicated
1219 antibodies. Protein abundance was calculated by normalization to GAPDH levels and
1220 plotted relative to the DMSO control. A representative blot is shown above the
1221 quantification of three independent experiments. Error bars represent SD.

1222 (C) ACH-2 cells were treated with ART and the indicated concentrations of EPL for 72 h
1223 and the IC₅₀ values calculated as in (A).

1224 (D) Cytotoxicity of SP (red) and EPL (blue) treated cells in ACH-2 cells treated for 72 h
1225 with increasing concentrations of SP was measured using an MTT assay and plotted as
1226 a percentage of the DMSO control. Error bars represent SD from three independent
1227 experiments.

1228 (E) ACH-2 cells were split and treated every 72 h in the presence of ART with 10 μM SP
1229 (red), EPL (light blue) or vehicle only (DMSO, dark blue). HIV capsid production was
1230 quantified by p24 ELISA. The dotted black line at 3 pg/ml represents the limit of
1231 detection (l.o.d). This plot is representative of three independent experiments.

1232 (F) Cell associated HIV mRNA levels (red) and *ERCC3* (XPB) mRNA levels (dark red)
1233 were measured at multiple time points during the long-term treatments of ACH-2 cells
1234 with 10 μM SP in (D). cDNA was prepared from RNA extracted from cells pellets and
1235 qPCR performed with primers in the Nef region. The results were normalized as the
1236 number of viral mRNA copies per RPL13A mRNA and plotted relative to the DMSO
1237 treated control (blue, set to 100% for each time point). Error bars represent SD from the
1238 qPCR.

1239 (G) The number of viable cells per ml of culture (left panel) and percentage viable cells
1240 (right panel), respectively, were measured by trypan blue staining and an automated
1241 cell counter every 72 h during the long-term treatments of ACH-2 cells with 10 μM SP,
1242 10 μM EPL or DMSO only.

1243 (H) HUT-78 cells were infected with SIV239 in the presence of 10 μM SP or 10 μM EPL
1244 (or vehicle only, DMSO). Capsid production was monitored over time by p27 ELISA.

1245 Inset is a representative Western blot of XPB protein expression 12 days post infection,
1246 the housekeeping gene GAPDH is included as a loading control.

1247 (I) Viability of the HUT-78 cells in (H) was measured by trypan blue staining using an
1248 automated cell counter. Error bars represent SD of two independent experiments.

1249

1250 **Figure 3. shRNA knockdown of the XPB gene, *ERCC3*, results in similar levels of**
1251 **HIV-1 inhibition in OM-10.1 cells.**

1252 OM-10.1 cells were transduced with pLKO.1 puro lentiviral vector expressing either a
1253 control shRNA targeting *GFP* mRNA or an *ERCC3* (XPB) shRNA. shGFP transduced
1254 cells were also treated with 1.8 μ M (IC₅₀), 6.3 μ M (IC₉₀) and 10 μ M SP. After 10 days of
1255 puromycin selection in the presence of ART and DMSO or indicated concentrations of
1256 SP cells were harvested for Western blot, p24 ELISA and RT-qPCR analysis.

1257 (A) Quantification of the XPB Western blots from three independent experiments. XPB
1258 expression was normalized to β -tubulin expression and plotted as a percentage of the
1259 shGFP control.

1260 (B) *ERCC3* (XPB) mRNA levels were measured by synthesizing cDNA from cell-
1261 associated RNA from transduced cells followed by RT-qPCR. Expression was
1262 normalized to RPL13A levels and plotted relative to the shGFP control.

1263 (C) The number of viable cells (left) and percentage of viable cells (right) were
1264 determined using trypan blue staining and an automated hemocytometer.

1265 (D) Cell associated HIV mRNA levels were quantified by RT-qPCR and normalized to
1266 RPL13A expression. Levels were plotted relative to the shGFP control.

1267 (E) The amount of HIV p24 capsid protein released into the supernatant of transduced
1268 OM-10.1 cells were measured by p24 ELISA.

1269 (F) Left panel: A representative Western blot of indicated proteins, from 3 independent
1270 experiments. The housekeeping genes GAPDH and β -Tubulin were included as loading
1271 controls. Right panel: Quantification of the XPD, CDK7 and Cyclin H Western blots,
1272 respectively, from three independent experiments. Protein expression was normalized
1273 to the respective housekeeping gene expression and plotted as a percentage relative to
1274 the shGFP control.

1275 Error bars represent the SD from three independent experiments. *P < 0.05, **P < 0.01,
1276 ***P < 0.001. ****P < 0.0001. NS unless indicated. One-way ANOVA followed by the
1277 Dunnett multiple comparisons test.

1278
1279

1280 **Figure 4. SP treatment blocks HIV reactivation.**

1281 (A) OM-10.1 cells were treated for 93 days with ART and 10 μ M SP or DMSO. Every
1282 three days the cells were split, treated and harvested for Western blot, p24 ELISA and
1283 RNA analysis. The amount of p24 capsid released into the supernatant of each culture
1284 was measured by p24 ELISA every three days.

1285 (B) ACH-2 cells were treated for 15 days with ART and 10 μ M SP or DMSO and treated
1286 as for OM-10.1 cells above (A).

1287 (C) After 45 days of treatment, OM-10.1 cells from (shown in A) were set aside from
1288 each long-term treatment with ART and 10 μ M SP (red) or DMSO (blue) and were
1289 stimulated with the indicated concentrations of LRAs for 8 h. The amount of cell

1290 associated HIV mRNA was determined by RT-qPCR, normalized to RPL13A levels and
1291 plotted relative to the unstimulated DMSO control.

1292 (D) The same was done on day 15 of long-term ACH-2 treatments with ART and 10 μ M
1293 SP or DMSO (shown in B).

1294 (E) Resting CD4⁺ T cells from 18 ART-treated, virally suppressed HIV-1 infected
1295 individuals were isolated from whole PBMCs. Patients had to have been on ART for > 1
1296 year with no blips in viral load in the past 6 months. Cells were treated with SP for 24 h
1297 in the presence of the fusion inhibitor, T20, to prevent new rounds of infection. In the
1298 final 6 h of treatment, cells were subjected to stimulation with PMA + Ionomycin. The
1299 amount of HIV-1 RNA was measured per million cells.

1300 (F) The amount of *POLR2A* mRNA was measured as in (E).

1301 The limit of detection (l.o.d.) is shown by the dotted line at 3.0 pg/ml. Error bars
1302 represent the SD from three independent experiments. *P < 0.05, **P < 0.01, ***P <
1303 0.001. ****P<0.0001. NS unless indicated. A paired T-Test was used to compare
1304 stimulated to unstimulated for each respective treatment. A Wilcoxon matched-pairs
1305 signed rank test was used to calculate the p-values for (E) and (F).

1306

1307 **Figure 5. SP treatment of OM-10.1 cells suppresses HIV in the absence of ART,**
1308 **but pre-treatment does not delay rebound upon treatment interruption.**

1309 (A) On day 69 of the long-term treatment of OM-10.1 cells with ART and 10 μ M SP or
1310 DMSO, some cells were set aside and washed to remove ART (indicated by the black
1311 arrows) and maintained alongside the treatments with ART. Cells were then treated
1312 every three days with either DMSO + ART (dark blue), 10 μ M SP + ART (red), DMSO

1313 with no ART (light blue) and 10 μ M SP with no ART (orange). The amount of HIV capsid
1314 released into the supernatant was monitored over time by p24 ELISA.

1315 (B) On day 78 of the long-term treatment of OM-10.1 cells with ART and 10 μ M SP or
1316 DMSO, some cells were set aside and washed to remove treatments with DMSO or SP
1317 (indicated by the black arrow). Cells were then treated every three days with either
1318 DMSO + ART (dark blue), 10 μ M SP + ART (red), ART with no DMSO (light blue) and
1319 ART with no SP (orange). The amount of HIV capsid released into the supernatant was
1320 monitored over time by p24 ELISA.

1321 (C) Cells were collected every three days from when DMSO or SP treatments were
1322 stopped (light blue and orange lines) for protein expression analysis by Western
1323 blotting. A representative blot of the indicated proteins is shown.

1324 The limit of detection (l.o.d.) is shown by the dotted line at 3.0 pg/ml. Error bars
1325 represent the SD from three independent experiments.

1326
1327

1328 **Figure 6. Treatment of OM-10.1 cells leads to a reduction RNA polymerase II**

1329 **occupancy along the HIV genome.**

1330 OM-10.1 cells on ~day 70 from long-term treatments with ART and 10 μ M SP or DMSO
1331 (Fig. 4A) were set aside and stimulated for 8 h with 1 μ M prostratin. Cells were then
1332 cross-linked prepared for Chromatin immunoprecipitation (ChIP).

1333 (A) ChIP to total RNAPII was followed by qPCR with primers specific to the indicated
1334 regions on the HIV genome (Table S2). The dark blue and red lines represent the
1335 distribution of RNAPII on the HIV genome in unstimulated cells. The light blue and
1336 orange lines represent the distribution of RNAPII on the HIV genome when cells were

1337 stimulated with 1 μ M prostratin for 8 h prior to crosslinking. Data for each point is plotted
1338 as a fold enrichment relative to the % of input for the unstimulated DMSO control with
1339 the first primer set. Grey asterisks mark significance comparing unstimulated SP treated
1340 to unstimulated DMSO treated cells. Black asterisks mark significance comparing
1341 stimulated SP treated to stimulated DMSO treated.

1342 (B) As above but using an antibody specific to RNAPII CTD phosphorylated on Ser5.

1343 (C) As above but using an antibody specific to RNAPII CTD phosphorylated on Ser2.

1344 (D) ChIP to total RNAPII was followed by qPCR with primers specific to the GAPDH
1345 promoter region or RPL13A ORF. Data for each point is plotted as a fold enrichment
1346 relative to the % of input for the unstimulated DMSO control with the first primer set.

1347 (E) As above but using an antibody specific to RNAPII CTD phosphorylated on Ser5.

1348 (F) As above but using an antibody specific to RNAPII CTD phosphorylated on Ser2.

1349 *P < 0.05, **P < 0.005, ***P < 0.0005. NS unless indicated. A two-tailed T-test was
1350 performed for the comparison of the different treatment groups in (A)-(C). One-way
1351 ANOVA followed by the Dunnett multiple comparisons test was used for (D)-(E). Error
1352 bars represent SD from three independent experiments.

1353

1354 **Figure 7: Effects of SP treatment on transcriptional machinery and cell cycle**
1355 **progression.**

1356 (A) OM-10.1 cells treated for 108, 114 and 123 days with ART and 10 μ M SP (red bars)
1357 or DMSO (blue bar) (Fig. 1D) were collected for analysis of mRNA expression of the
1358 indicated components of TFIIH. cDNA was prepared from cell-associated RNA and
1359 qPCR performed. The results were normalized per RPL13A and plotted relative to the

1360 DMSO treated control (set to 100%, dotted line). Error bars represent the SD from three
1361 independent timepoints.

1362 (B) Left panel: A representative of three independent Western blots of the indicated
1363 proteins from OM-10.1 cells treated 108, 114 and 123 days with ART and DMSO, 10
1364 μM SP or 10 μM EPL. Right panel: Quantification of the Western blots on the left,
1365 protein expression of the indicated genes were normalized to the housekeeping gene,
1366 GAPDH or β -tubulin, and expressed relative to the corresponding DMSO treated control
1367 (set to 100%, dotted line).

1368 (C) Left panel: A representative Western blot of the indicated genes in ACH-2 cells
1369 treated for 15 days with ART and 10 μM SP or DMSO only. Right panel: Western blot
1370 quantification of three independent experiments. The protein expression levels were
1371 determined by quantification of the blots, normalized to the housekeeping gene β -
1372 tubulin, and expressed relative to the DMSO treated control (set to 100%, dotted line).

1373 (D) Left panel: A representative Western blot of three independent experiments
1374 measuring the protein expression of the indicated genes over time (d0, d3, d6 and d21).
1375 OM-10.1 cells were treated with ART + 10 μM SP or DMSO for the indicated number of
1376 days before harvesting cells for Western blot. Right panel: Quantification of the Western
1377 blots on the left for the indicated proteins, levels were normalized to the housekeeping
1378 gene, GAPDH or β -tubulin, and expressed relative to the corresponding DMSO treated
1379 control (set to 100%).

1380 (E) OM-10.1 cells were treated with ART + DMSO or 10 μM SP for 24 days after which
1381 cells were collected for Western blot analysis of the indicated proteins. Left panel: A
1382 representative Western blot of 3 independent experiments. Right panel: Quantification

1383 of the three Western blots, protein expression of the indicated genes were normalized to
1384 the housekeeping gene, GAPDH or β -tubulin, and expressed relative to the
1385 corresponding DMSO treated control (set to 100%).

1386 (F) OM-10.1 cells were treated with ART and either DMSO, 10 μ M SP or 10 μ M EPL for
1387 ~80 days. Cell cycle stage analysis was performed by propidium iodide staining and
1388 flow cytometry 72 h post treatment. Left panel: A representative plot showing the degree
1389 of PI staining under each treatment condition. Right panel: A summary of the % of cells
1390 in the indicated stage of the cell cycle.

1391 Error bars represent the SD from three independent experiments. *P < 0.05, **P < 0.01,
1392 ***P < 0.001. ****P<0.0001. NS unless indicated. One-way ANOVA followed by the
1393 Dunnett multiple comparisons test.

1394

1395 **Figure 8. Potent inhibition of HIV mRNA expression with limited effects on cellular**
1396 **transcription.**

1397 OM-10.1 cells were treated with ART+DMSO or ART+10 μ M SP for 15 days (until HIV
1398 capsid production in supernatant in SP-treated cells stabilized below the limit of
1399 detection by p24 ELISA) followed by an 8 h stimulation (or not) with 1 μ M prostratin.
1400 mRNA-seq and differential expression analysis was performed on these cells. Data are
1401 from two biological replicates.

1402 (A) Volcano plots showing differentially expressed genes under the indicated conditions.
1403 HIV encoding genes are colored red and the most significantly differentially expressed
1404 genes are colored blue [pAdj<1e-10 & abs(log2FoldChange)>1 for SP vs DMSO
1405 comparisons (left and right panels), and padj<1e-30 with an abs(log2FoldChange)>2.5

1406 for Stim vs No stim comparisons (middle two panels)]. The horizontal dotted line
1407 represents a $p_{Adj} = 0.05$ and the vertical dotted lines a \log_2FC of 1 and -1. Left panel:
1408 The change in expression of all genes (dots) in SP treated compared to DMSO treated
1409 cells without stimulation. Middle left panel: Differentially expressed genes in DMSO
1410 treated cells stimulated with prostratin compared to unstimulated cells. Middle right
1411 panel: Differentially expressed genes in SP treated cells stimulated with prostratin
1412 compared to unstimulated cells. Right panel: Differentially expressed genes in prostratin
1413 stimulated cells treated with SP compared to DMSO.

1414 (B) GSEA Bubble-Lattice Plot of selected gene sets. The color represents the
1415 normalized enrichment score (NES) indicating the number and differential intensity of
1416 the assessed genes in each indicated pathway. $NES > 0$: the gene set is enriched in the
1417 SP treated sample (red). $NES < 0$: the gene set is enriched in the DMSO treated sample
1418 (blue). NS = unstimulated, Stim = prostratin stimulated. The size of the dots indicates
1419 the statistical significance of the enrichment found (log transformed familywise error
1420 rate, $-\log_{10}$ FWER p value). GSEAs were computed from the GSEA app v4.1.0 using
1421 TPMs as counts and publicly available gene sets current as of October 2020 (71).

1422 (D) Heat map of expression levels (transcript per kilobase million (TPM) values) for
1423 selected genes with DMSO or SP treatment, with and without stimulation with prostratin.















