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1 Direct tracking of single proviruses reveals HIV-1/LEDGF 2 complexes excluded from virus-induced membraneless 3 organelles

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17
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19 Running title: HIV-1 MLOs retain integrase and release viral DNA

20 Abstract

21 The ultimate goal of HIV-1 is integration into the host chromatin to optimize the release
22 of high levels of viral progeny and discretely coexist with the host. To uncover the HIV-
23 1 DNA fate in the nuclear landscape we directly tracked the viral DNA (vDNA) and the
24 viral RNA (vRNA) by coupling HIV-1 ANCHOR technology with RNA FISH or MCP-
25 MS2 RNA-tagging bacterial system. Our computational imaging analysis revealed that
26 proviral forms are early located in proximity of the nuclear periphery of mitotic and non-
27 mitotic cells. We also observed that HIV-1 infection prompts clustering formation of the
28 host factor CPSF6 restructuring membraneless organelles enriched in both viral
29 proteins and speckle factors. Interestingly, we observed that integrase proteins are
30 retained in CPSF6 clusters, while the late retrotranscribed DNA was excluded from
31 HIV-induced membranellless organelles (HIV-1 MLOs), indicating that those structures
32 are not proviral sites, but orchestrate viral events prior to the integration step. HIV-1
33 MLOs are in the vicinity of pre-existing LEDGF clusters. Importantly, we identified
34 actively transcribing proviruses localize, outside HIV-1 MLOs, in LEDGF-abundant
35 regions, known to be active chromatin sites. This study highlights single functional
36 host-proviral complexes in their nuclear landscape, which is markedly restructured by
37 HIV-1 to favor viral replication.

38

39 **Introduction**

40 Immediately after fusion at the plasma membrane, HIV-1 cores are released into the
41 cytoplasm and move towards the nucleus, while the viral RNA genome begins the
42 process of reverse transcription into double-stranded DNA (dsDNA) (Campbell and
43 Hope, 2015; Di Nunzio, 2013). Once imported in the nucleus, the interplay between
44 the HIV-1 DNA genome and the host chromatin compartment is crucial for the fate of
45 the virus-host coexistence. HIV-1 can adopt either an episomal or a proviral form
46 (Butler et al., 2001). The choice between these two possibilities and their surrounding
47 chromatin landscape dictates the evolution of HIV infection (Maldarelli, 2016; Sharkey
48 et al., 2011; Sharkey et al., 2000). The mechanisms determining the fate of the proviral
49 DNA in the nucleus are still under investigation (Liu et al., 2020; Olson et al., 2019)
50 also because of the limits imposed by available technologies. Real-time imaging
51 approaches provide new insight into unprecedented spatial information of individual
52 HIV-1 infected cells. Several strategies have enabled the visualization of the viral
53 components during nuclear entry: Integrase (IN)-TC/FIAsH, APOBEC 3F (A3F)-YFP,
54 IN-YFP, Gag-iGFP, Cyclophilin A (CypA)-DsRed/ Capsid (CA), INsfGFP, and CA-GFP
55 (Burdick et al., 2017; Hubner et al., 2009; Lelek et al., 2012) (Francis et al., 2016;
56 Francis and Melikyan, 2018) (Mamede et al., 2017) (Albanese et al., 2008; Francis et
57 al., 2014). Previously, biochemistry studies supported only a limited and secondary
58 role of the viral CA in the early steps of infection (Farnet and Haseltine, 1991). State-
59 of-the-art imaging technologies developed in recent years have shed light on the
60 central role of the viral CA in nuclear import (Blanco-Rodriguez et al., 2020; Burdick et
61 al., 2020) dictated by the CA interplay with host factors, such as CPSF6 (Buffone et
62 al., 2018; Francis et al., 2020; Price et al., 2014) and Nup153 (Di Nunzio et al., 2013;
63 Lelek et al., 2015). Although these techniques improved our knowledge in the HIV field,
64 the labeling of viral proteins only allows the visualization of steps prior to the
65 establishment of proviral or episomal forms. Mechanisms underlying viral post-nuclear
66 entry steps and the role of nuclear structures induced by HIV-1 during viral replication
67 could be revealed only by the direct co-labeling of viral DNA (vDNA) forms and host
68 factors. Thus far, the vDNA can be visualized either by supplying 5-Ethynyl-2'-
69 deoxyuridine (EdU) during infection (Peng et al., 2014), which is limited to fixed non-
70 dividing cells (De Wit et al., 2019) or by DNA-FISH (Marini et al., 2015), which is
71 characterized by harsh conditions of fixed sample preparation that limits its coupling

72 with other techniques, impeding the study of the interplay between the vDNA and the
73 host factors. Here, we were able to live track episomal and proviral forms, showing
74 their divergent intranuclear behavior. In parallel, we detect the location of functional
75 proviruses in the nuclear space of the main target cells of the virus, such as CD4⁺T
76 cells and macrophages, by using HIV-1 ANCHOR methodology (Blanco-Rodriguez et
77 al., 2020) to label the vDNA coupled to RNA FISH or to the live bacterial approach
78 MCP-MS2 (Tantale et al., 2016) to identify the vRNA foci. Next, we deepened the study
79 of the nuclear landscape that surrounds proviruses. We observed that viral infection
80 reprogramed the nuclear location of a viral partner, CPSF6. CPSF6 is a paraspeckle
81 factor (Fox et al., 2002; Naganuma and Hirose, 2013) involved in viral nuclear entry
82 though its interaction with the viral CA (Buffone et al., 2018; Francis et al., 2020; Lee
83 et al., 2010; Price et al., 2014). Here we found that CPSF6 relocates in HIV-1 induced
84 membraneless organelles (HIV-1 MLOs), enriched in speckle factors, SC35 (Spector
85 and Lamond, 2011) and SON (Sharma et al., 2010), near LEDGF clusters. Previous
86 studies reported the importance of LEDGF in HIV-1 integration sites distribution (Ciuffi
87 et al., 2005), however a complex between the transcribing proviral DNA and LEDGF
88 has never been visualized. In this study, we determined the nuclear position of several
89 viral forms, including actively transcribing proviruses, with respect to HIV-1- MLOs or
90 the nuclear envelope (NE). In addition, we found that the late retro-transcribed DNA
91 co-exists, but remains separated, from viral INs in the host nucleus. We observed that
92 the viral INs are sequestered in HIV-1 MLOs, suggesting the role of these nuclear
93 structures in the Pre-Integration Complex (PIC) maturation. On the other hand, the late
94 reverse transcripts are fully excluded from HIV-1 MLOs, in fact we found that HIV-1
95 MLOs are not the sites of viral integration. Finally, we identified functional host-viral
96 complexes, formed by vDNA/vRNA associated to LEDGF, excluded from the inner
97 cavity of HIV-1 MLOs (avg distances from HIV-1 MLOs < 0.6 μ m) and located in the
98 vicinity of the NE (avg of distances from NE < 2 μ m). In this study we show that HIV-1
99 DNA localization is key to identifying partners of HIV-1 that aid the virus to hijack
100 cellular mechanisms to persist in the host cells and to highlight the nuclear landscape
101 surrounding single viral genomes. Our results provide new insights into how HIV
102 reprograms and markedly restructures the nuclear environment to orchestrate viral
103 replication steps.

105 **Results**

106 **Live tracking of HIV-1 DNA forms in the nucleus of target cells.**

107 With the aim of studying HIV-1 DNA nuclear fate in different cell types, we benefited of
108 the HIV-1 ANCHOR DNA labeling system (Fig. 1A) (Blanco-Rodriguez et al., 2020).
109 Once fully retrotranscribed, the HIV-1 genome carrying the bacterial sequence ANCH3
110 is specifically recognized by OR-GFP protein, which is expressed in the target cells,
111 genetically modified by the lentiviral vector (LV) OR-GFP (Blanco-Rodriguez et al.,
112 2020). The accumulation of OR-GFP, which is a modified version of the bacterial ParB
113 protein (Graham et al., 2014; Sanchez et al., 2015), on the ANCH3 sequence
114 generates bright nuclear signals (Fig.1A). Importantly, OR protein binds exclusively
115 double stranded DNA (Saad et al., 2014). We cloned ANCH3 in the nef gene, thus,
116 HIV-1 ANCHOR system exclusively detects late reverse transcripts, because only late
117 retrotranscripts contain ANCH3 sequence as double stranded DNA (Fig. 1A).
118 Therefore, HIV-1 ANCHOR system allows only the visualization of potential functional
119 vDNA. We optimized and set up our imaging approach in HeLa P4R5 cells (Charneau
120 et al., 1994), a widely used cellular model for HIV-1 infection. Previous studies showed
121 that HeLa cells enable single particle tracking of HIV-1 proteins (Burdick et al., 2020;
122 Francis and Melikyan, 2018). However, since HIV-1 genome dissociates from viral
123 proteins to become a proviral DNA or an extrachromosomal DNA, the track of these
124 viral forms is the only direct measurement to investigate the fate of the viral genome
125 into the host nuclear space. Unlike previous studies, we are now able to directly
126 visualize by live imaging the nuclear vDNA as GFP puncta appearing in the host
127 nucleus once retrotranscribed (Fig. 1A). On the contrary, bright individual spots are
128 rarely visualized in the cytoplasm, where the vDNA is only partially accessible to OR-
129 GFP, as we previously demonstrated (Blanco-Rodriguez et al., 2020). HIV-1 ANCHOR
130 is able to specifically label the vDNA as shown by the correlation between the nuclear
131 GFP puncta and the MOI (multiplicity of infection) used (Fig. S1A). This specificity was
132 corroborated using the reverse transcription inhibitor, Nevirapine (NEV), which
133 completely prevents the detection of vDNA (Fig. 1B, S1B). We then investigated
134 whether HIV-1 ANCHOR allows the detection of different intranuclear vDNAs, such as
135 episomal and integrated forms. To generate episomal forms, either we infected cells
136 with HIV-1 ANCH3 in the presence of the integration inhibitor Raltegravir (RAL), or
137 using an integration-deficient virus, which is mutated at the catalytic site of the

138 integrase (IN) enzyme (HIV-1 ANCH3 IN_{D116A}). We detected intranuclear GFP puncta
139 under both conditions (Fig. 1B), meaning that HIV-1 ANCHOR permits the detection of
140 nuclear episomal vDNA forms. The experiments were validated by qPCR, which
141 confirmed the extrachromosomal forms generated during infection when the
142 integration step is impeded (Fig. 1B). We next assessed whether HIV-1 ANCHOR is
143 sufficiently sensitive to allow the detection of integrated vDNA at the level of a single
144 provirus. We selected single HeLa P4R5 clones carrying the HIV-1 ANCH3 genome.
145 The imaging data correlates with results obtained by ALU PCR (Fig. 1C),
146 demonstrating that this technology is powerful enough to detect a single provirus.
147 Thus, we used this system to live track proviruses and episomal forms to investigate
148 their behaviour in the nucleus (movies S1, S2, and S3). We performed live tracking of
149 vDNAs in cells at six days post infection (p.i.). (movie S1), which contain a negligible
150 amount of episomal forms, likely lost during cell division (Fig. S1C), in the single-cell
151 clone (movie S2) or in cells containing only the episomal vDNA (movie S3). Then, we
152 compared vDNA trajectories in the aforementioned experimental conditions (Fig. 1D).
153 The stable clone and the cells at six days p.i. show vDNA with similar trajectories and
154 diffusion coefficient (D (nm²/s) < 5). Instead, infected cells carrying unintegrated
155 viruses harbored two populations composed of episomal forms with divergent
156 behaviors, one with a low D (D (nm²/s) < 5) and the other with a high D ($5 < D$ (nm²/s)
157 < 20) (Fig. 1D). Our data hint that most, but not all, episomal forms are retained in
158 particular nuclear regions, most likely interacting with host factors or chromatin
159 compartments, consistent with the findings of other studies (Geis and Goff, 2019; Zhu
160 et al., 2018), whereas some episomal forms are free to move in the nuclear space. On
161 the other hand, the integrated forms showed a uniform, low-diffusive behavior.

162

163 **Detection of actively transcribing viral DNA at single-cell level in HIV-1 target** 164 **cells.**

165 The identification of functional vDNA in the nucleus of infected cells has been one of
166 our goals. For this purpose, we combined HIV-1 ANCHOR technology with RNA FISH
167 (single molecule inexpensive FISH, smiFISH) against HIV-1 polymerase transcripts, to
168 detect vRNA foci of transcription (Tsanov et al., 2016). As expected, in cells treated
169 with RAL, where the integration step is blocked, vRNA signal is highly depleted. In
170 untreated cells it is possible to observe vDNA-vRNA associations, highlighting the
171 proviruses location (Fig. 2A), in fact proviruses are the only transcribing forms. We

172 strengthen our results on the ability to visualize proviruses by showing the vDNA-vRNA
173 association by live imaging. HIV-1 transcriptional activity was tracked by MCP-GFP
174 system (kind gift from E. Bertrand) to label vRNA (Tantale et al., 2016) coupled with
175 HIV-1 ANCHOR technology (Fig. S2A, B). Next, we compared the intensity of
176 ANCHOR signals of the episomal forms (RAL) with those of the integrated forms
177 (untreated cells) and observed that the signal of unintegrated DNA was two-fold
178 brighter than the proviral DNA spot (Fig. 2A). Interestingly, we also remarked, during
179 live imaging, that the vDNA of the integration-deficient virus (HIV-1 ANCH3 IN_{D116A})
180 aggregated to form “flower-shaped structures” in the nucleus (Fig. S3, movie S4).
181 Aggregation of extrachromosomal forms could prompt a brighter appearance of viral
182 episomal DNA compared to a dimmer signal of the individual proviral DNA (Fig. 2A).
183 Next, we tested the HIV-1 ANCHOR system in the context of the main target cell of
184 HIV-1, primary CD4⁺ T cells, derived from healthy donors. CD4⁺ T cells challenged with
185 various MOIs of HIV-1 ANCH3 showed that the number of nuclear spots increased
186 with the increasing of the MOI (Fig. S4), meaning that HIV-1 ANCHOR specifically
187 detects the vDNA in the nucleus of primary lymphocytes. The correlation between
188 different MOIs used and the number of proviruses ($r=0.9412$), analysed by qPCR,
189 indicates that OR-GFP does not impede the viral integration step (Fig. S4). We tested,
190 as well, the co-detection of viral transcriptional foci and vDNA in T lymphocytes
191 (primary CD4⁺ T and Jurkat cells) through RNA FISH (Fig. 2B, Fig. S5). We observed
192 that not all vDNAs associated to a transcriptional focus at 48 h p.i., most likely because
193 of the presence of silent episomal forms. On the other hand, the majority of cells
194 analyzed contain vRNA foci colocalized with vDNA (Fig. 2B), meaning that we are able
195 to detect single transcribing proviruses. We also performed similar experiments in non-
196 dividing cells, THP-1, which are macrophage-like cells. Thus, we imaged and analyzed
197 THP-1 cells at 3 days p.i. and we observed that also in these cells we could distinguish
198 individual transcribing proviruses from silent viral forms (Fig. 2C). Of note, the RNA
199 signal considered in our images was exclusively due to the detection of viral
200 transcription foci, since samples treated with NEV did not show any significant intra-
201 nuclear signal (Fig.S6). Taken together, our results suggest that we are able to
202 distinguish episomal forms (mainly silent) from integrated forms that are the only ones
203 able to replicate.

204 **Nuclear distance of actively transcribing proviruses from the nuclear envelope**
205 **at single-cell level in dividing and non-dividing cells.**

206 The location of actively transcribing proviruses in the nuclear space of dividing cells is
207 still a subject of debate (Achuthan et al., 2018; Burdick et al., 2017; Chin et al., 2015;
208 Di Primio et al., 2013; Lelek et al., 2015; Marini et al., 2015), whereas their nuclear
209 location in terminally differentiated cells, as far as we know, has never been studied
210 before. In dividing cells, the 3D analysis of the nuclear location of the vDNA showed
211 the vDNA signals at 48 h p.i. to be more randomly distributed throughout the nucleus
212 compared to the vDNA spots at seven days p.i. (Avg distances from the NE: $\sim 2.5 \mu\text{m}$
213 at 48 h p.i. vs $\sim 1.4 \mu\text{m}$ at 7 days p.i.) (Fig. 3A). The difference in the average distance
214 of the vDNA spots from the NE between the early and late time points p.i. may be due
215 to the co-existence of heterogeneous viral forms at 48 h p.i. (episomal and proviral
216 forms), whereas the vDNA population is more homogeneous at seven days p.i. (Fig.
217 1D, 3A). In fact, at seven days p.i., almost all remaining vDNAs in the nucleus consisted
218 of integrated forms, as suggested by the imaging of dividing cells following prolonged
219 treatment with RAL (Fig. S1C). Interestingly, we did not detect differences between the
220 distances of the vRNA foci from the NE ($\sim 1.5 \mu\text{m}$) at both time points (48h p.i. and 7d
221 p.i.) and the vDNA spots at seven days p.i. ($\sim 1.4 \mu\text{m}$), indicating that in dividing cells
222 the integration of the proviral DNA occurs early during infection near to the NE (Fig.
223 3A). We performed similar 3D analysis to calculate the distances of vDNAs and vRNAs
224 from the NE in non-dividing cells at three days p.i.. The lack of mitosis hinders the loss
225 of the episomal forms in non-dividing cells, so we could not discriminate between
226 integrated and unintegrated vDNA forms. However, when we compared vDNAs
227 (episomal and proviral forms) versus vRNA foci (actively transcribing proviral forms)
228 their analysis of distances from the NE revealed a major vicinity of the proviruses to
229 the NE compared to the total vDNA ($\sim 2.2 \mu\text{m}$ for the vDNA vs $\sim 1.8 \mu\text{m}$ for the vRNA)
230 (Fig.3B). Overall, our data show a similar nuclear spatial distance of actively
231 transcribing proviral forms from the NE at single-cell level in both, dividing and non-
232 dividing cells (distance of vRNA foci from the NE: $\sim 1.7 \mu\text{m}$ in HeLa cells and $\sim 1.8 \mu\text{m}$
233 in THP-1 cells) (Fig.3C).

234 **The majority of IN proteins are retained in CPSF6 clusters and separate from the**
235 **late retrotranscribed DNA**

236 A long-standing question in the early steps of HIV life cycle is whether viral IN proteins
237 remain all associated with late reverse transcribed DNA for long time or only for a short
238 time after the nuclear entrance. This association may not be detectable because of the
239 short-time nature of the event or due to the technical limitations of vDNA and IN
240 visualization. Thus, we exploited our vDNA tracking system to investigate about IN
241 proteins localization in primary CD4⁺T cells in comparison to terminally differentiated
242 cells, THP-1. We challenged both cell types with HIV-1 ANCH3 and we analysed them
243 at 20h and 30h p.i., respectively. We found that in both cells the vDNA/IN association
244 event was very rare (only one single event in 1 cell for THP-1 and in 3 cells for CD4⁺T
245 cells) (Fig. 4A). Nuclear IN protein clusters were found in the nucleus, but not at the
246 site of the late reverse transcripts (Fig. 4A). To confirm our data we performed live-
247 imaging, asking whether we could pinpoint the dynamic evolution of the association
248 between the vDNA and the IN in the nucleus of infected cells by coupling the HIV-1
249 ANCHOR system with the GIR virus (generous gift from Edward Campbell)(Dharan et
250 al., 2016; Hulme et al., 2015). We produced the GIR virus using a viral genome carrying
251 the IN with a mutation in the catalytic site (HIV-1 ANCH3 IN_{D116A}), as consequence,
252 the only active IN in these viral particles was the IN-Ruby (Fig. S7). Analysis of the
253 dynamics of the vDNA / IN association shows the two signals to be separated (~ 0.6
254 to 0.8 μ m) within the nucleus for several hours (Fig. S7, movie S5). Our results suggest
255 that, after nuclear entry, the main localization of IN is not at the exact site of the viral
256 DNA forms, even though we can sometimes spot them in proximity. Next, we
257 investigated whether the viral IN was retained in particular nuclear regions by some
258 nuclear host factors. We first found that the viral IN occupies the same positions as the
259 viral CA and its host nuclear partner, CPSF6 (Fig. S8A). We confirmed that HIV-1
260 reprograms the nuclear localization of CPSF6 from being randomly dispersed in the
261 nucleoplasm to form particular aggregates in both primary lymphocytes and
262 macrophages (Francis et al., 2020) (Rensen et al., BioRxiv
263 doi.org/10.1101/2020.04.12.038067) (Fig. 4B). We also observed that CPSF6-IN-CA
264 clustering is independent of DNA synthesis (Fig. S8B). Then, we quantified the
265 percentage of association per nucleus between the viral IN and CPSF6. We found an
266 average of association of IN to CPSF6 ~ 94.4% in CD4⁺T cells and ~92.5% in THP-1
267 cells (Fig. 4B). Taken together our results show that in dividing and in non-dividing
268 cells there is an intra-nuclear separation of the viral DNA and of the IN proteins.
269 Interestingly, the viral IN proteins persist after viral nuclear entry associated to CPSF6

270 clusters. This observation suggests that the late reverse transcripts are released from
271 the majority of IN proteins which instead are retained in a separated nuclear location
272 occupied by CPSF6. Thus, CPSF6 clusters could serve as sites to release a mature
273 PIC that could contain just few IN proteins necessary for the integration step
274 (Ballandras-Colas et al., 2017; Hare et al., 2009).

275 **Provirus are excluded from HIV-1-induced membraneless organelles.**

276 CPSF6 participates to the viral nuclear translocation and post-nuclear entry steps by
277 interacting with the viral CA (Bejarano et al., 2019; Burdick et al., 2017; Lee et al.,
278 2010; Price et al., 2014) (Francis et al., 2020). Recent studies reported a high level of
279 co-localization between the paraspeckle factor, CPSF6, or the speckle factor and
280 splicing regulator, SC35, (Spector and Lamond, 2011) and the vDNA labeled with EdU
281 (Francis et al., 2020) (Bejarano et al., 2019)(Rensen et al., BioRxiv
282 doi.org/10.1101/2020.04.12.038067). Importantly, it has been shown that EdU labels
283 nuclear clusters composed of multiple viral episomal forms (Francis et al., 2020)
284 (Rensen et al., BioRxiv doi.org/10.1101/2020.04.12.038067). Therefore, EdU labeling
285 can detect multiple nascent vDNAs but it is likely not sufficiently sensitive to detect an
286 individual viral genome. Contrary to EdU labeling, the HIV-1 ANCHOR system allows
287 the visualization of a single provirus (Fig.1C) and does not interfere with viral
288 transcription (Blanco-Rodriguez et al., 2020) (Rensen et al., BioRxiv
289 doi.org/10.1101/2020.04.12.038067). Thus, we used HIV-1 ANCHOR technology to
290 visualize the late retrotranscripts in infected macrophage-like cells at 3 days p.i.. We
291 observed that late retrotranscripts do not colocalize with CPSF6 (Fig. 5A). However,
292 upon viral infection, CPSF6 relocates and fully colocalizes with speckle-simile
293 organelles labeled with SC35 (Fig. 5A). SC35 is strongly associated to another speckle
294 factor, SON, independently of viral infection (r pearson ~0.995). More specifically, we
295 found that the nuclear reorganization of CPSF6 is not required for the integration step,
296 as the nuclear organization of CPSF6 was similar in cells treated or not with RAL (Fig.
297 S9). Then, we investigated whether the vDNA colocalizes with SC35. We computed
298 vDNA distances in 3D space from the boundary of SC35 bodies in cells treated with
299 RAL (avg ~ 0.4 μm (Fig. S9)) or in absence of RAL (avg ~ 0.6 μm (Fig. 5A)). Therefore,
300 similarly to CPSF6 also SC35 clusters exclude late reverse transcripts (Fig. 5A). In
301 addition, we found that viral transcriptional foci where also located outside
302 CPSF6/SC35 organelles (Fig. 5B). These results are independent of the presence of

303 vpx (Fig. S10) that we used to increase viral infectivity in THP-1 (Hrecka et al., 2011;
304 Laguette et al., 2011). Taken together analysis of the nuclear positions of vDNA
305 (episomal and proviral forms) and vRNA foci (proviruses) showed that these viral
306 nuclear forms are excluded from HIV-1 MLOs (Fig. 5C), indicating that those
307 organelles cannot be sites for viral integration or replication.

308

309 **Late reverse transcripts DNA and viral RNA foci form complexes with LEDGF,**
310 **spatially independent of CPSF6 clusters.**

311 We extended our study on THP-1 cells at 3 days p.i. to investigate whether Lens
312 Epithelium-Derived Growth Factor (LEDGF), which is a partner of the viral IN and leads
313 HIV-1 integration sites selection (Cherepanov et al., 2003; Ciuffi et al., 2005; Emiliani
314 et al., 2005; Ferris et al., 2010; Hare et al., 2009; Lelek et al., 2015; Llano et al., 2006;
315 Shun et al., 2008; Shun et al., 2007; Wang et al., 2014), is associated to the proviruses
316 forming a complex visible by imaging. We observed that CPSF6 proteins cluster in
317 proximity of LEDGF sites (~ 43%), without co-localizing (Fig. 6A). There is a similar
318 number of LEDGF clusters in both infected and uninfected cells (Fig. 6A), while CPSF6
319 clusters are mainly formed upon infection, suggesting that CPSF6 is prompted by the
320 virus to localize near the pre-existing LEDGF regions (Fig.6A). Of note, the PWWP
321 domain of LEDGF has been shown to interact with H3K36me3 (Pradeepa et al., 2014;
322 Pradeepa et al., 2012), which is a marker of euchromatin. In particular, this post-
323 translational modification is enriched in HIV-1 integration sites (Ciuffi et al., 2005; Lelek
324 et al., 2015; Vansant et al., 2020; Wang et al., 2009). LEDGF also interacts with
325 splicing factors (Singh et al., 2015). Therefore, we analysed the location of proviral foci
326 of transcription in relation to LEDGF and CPSF6. Our previous data shows that CPSF6
327 co-localizes with SC35, however the distance between the vDNA and SC35 has been
328 estimated to be ~ 0.6 μm (Fig. 5A), therefore, the late retrotranscribed DNA is too
329 distant from those HIV-1 MLOs to be considered part of the same complex. While, the
330 vDNA of the late retrotranscripts is mostly excluded from CPSF6 clusters, we found
331 that ~64% of the vDNA and ~73% of the vRNA foci associate with LEDGF (Fig. 6B).
332 Next we have characterized in more detail the viral genome associations with LEDGF
333 and CPSF6. We calculated that ~ 87.9% of the vDNA/LEDGF complexes and ~80%
334 of vRNA foci/LEDGF complexes are uncoupled to CPSF6 (Figure 6B). We found only
335 few vDNA (~12.1%) and vRNA (~20%) foci associated to LEDGF clusters coupled to
336 CPSF6 (Fig. 6B). Our results suggest that the two cellular factors, CPSF6 and LEDGF,

337 orchestrate two sequential steps of viral replication, the maturation of the PIC and its
338 integration. Our data indicate that the functional vDNAs are not located inside
339 CPSF6/SC35 clusters, but in external active chromatin domains, enriched in LEDGF,
340 likely to benefit of a favorable environment for viral replication.

341

342 **Discussion**

351 Studies of parasites that permanently integrate into the host genome show that they
352 have evolved to target chromatin in such a way as to optimize their coexistence with
353 the host and favor the release of new viral progeny (Bushman, 2003; Craig and
354 Marszalek, 2002). HIV-1 needs to rapidly release high levels of newly generated
355 viruses after infection, intuitively explaining the preference of active genes as
356 integration targets (Ciuffi et al., 2005; Di Nunzio et al., 2013; Lelek et al., 2015;
357 Maldarelli, 2016; Schroder et al., 2002). However, the nuclear location of individual
358 proviruses actively transcribing at the dividing or non-dividing single cell level has
359 never been visualized before. Thus far, the direct detection of HIV-1 genomes after
360 reverse transcription and the distribution of proviral transcriptional foci has been
361 technically challenging. Recent studies have shown the possibility to follow HIV
362 infection in live using surrogate viruses (IN GFP virus (Albanese et al., 2008), a mix of
363 viruses carrying fluorescent and non-fluorescent IN proteins (Hulme et al., 2011), or
364 indirectly follow the viral journey by labeling host viral partners, such as CypA (Francis
365 and Melikyan, 2018) or APOBEC 3F (Burdick et al., 2017)). All these approaches allow
366 to follow viral or host proteins but not viral replication complexes. HIV-1 ANCHOR
367 system allows the live tracking of different nuclear late retrotranscribed DNA forms
368 (Fig.1A), which makes possible to probe their behavior via the measurement of the
369 mean-squared displacement (MSD). We observed a homogenous population formed
370 by proviral DNA with low diffusion coefficient. Episomal forms showed a
371 heterogeneous behavior, characterized by two different populations, implying that
372 these two viral forms can interact with different factors in the nuclear space (Fig. 1D).
373 Moreover, imaging analysis showed that viral episomal forms identified by the
374 accumulation of OR-GFP on ANCH3 sequences gave rise to brighter signals than
375 proviral forms (Fig. 2A). This phenomenon could account for the differences in
376 behavior between integrated and unintegrated forms. Thus, unintegrated forms can be
377 brighter for the following reasons: a) the absence of transcriptional machinery

378 associated with the vDNA may reduce the steric hindrance that can interfere with the
379 accumulation of OR-GFP on the HIV-1 tag sequence (ANCH3) (Fig. 2A), b) episomal
380 forms can aggregate in a flower shape, generating a brighter signal (Fig. S3, movie
381 S4), c) episomal forms are differently chromatinized than the integrated vDNAs,
382 becoming more accessible to OR-GFP (Geis and Goff, 2019). Next, to track the
383 nuclear location of functional proviruses we co-labeled vDNA and vRNA by combining
384 HIV-1 ANCHOR technology with RNA FISH in the main target cells of HIV-1, CD4⁺T
385 cells and macrophages (Fig.2A,B,C). We also corroborated results on the visualization
386 of functional proviruses using a live track method, MCP-MS2 system (Tantale et al.,
387 2016), coupled to HIV-1 ANCHOR (Fig.S2). Once the performance of our approach for
388 labeling actively transcriptional proviruses was validated, we investigated the spatial
389 location of proviruses in dividing and non-dividing cells. Three-dimensional analysis of
390 the distribution of vDNAs during early (48 h p.i.) infection in mitotic cells displays that
391 they are more randomly distributed than the proviral DNA from the single clone or from
392 cells at 7days p.i. (Fig. 3A). We also observed that the location of viral transcription
393 sites (vRNA) is highly similar between early and late time points of infection, indicating
394 that proviral DNAs, the only transcribing forms (Fig. 2A), immediately localize to near
395 the NE (~1.5 μ m) early after infection (Fig. 3A) in dividing and non-dividing cells
396 (Fig.3B,C). We next investigated the interplay among viral components and their
397 association with host factors during post-nuclear entry steps. We corroborated our
398 published data (Rensen et al.,BioRxiv doi.org/10.1101/2020.04.12.038067) showing
399 that IN proteins strongly co-localize with CPSF6 clusters in macrophage-like cells and
400 in this study we observed a similar association between IN and CPSF6 also in CD4⁺ T
401 lymphocytes at early time of infection (Fig. 4B). Contrary to CPSF6, the late vDNA
402 products of retrotranscription rarely co-localize with IN in both types of cells (Fig. 4A).
403 These results are in line with the dynamic coexistence of the IN and vDNA, which
404 remain separated but in the proximity for several hours, as it has been shown by real-
405 time imaging in HeLa cells (Fig. S7, movie S5). This result can appear in contrast with
406 recent data from other groups and from us obtained with another DNA labeling
407 technology, EdU (Francis et al., 2020) (Rensen et al., BioRxiv, 2020), showing the
408 vDNA and the IN strongly colocalized with CPSF6. This apparent discrepancy can be
409 explained by the intrinsic features of vDNA labeling tools. EdU system is capable of
410 detecting nascent episomal forms of vDNA abundant in CPSF6 clusters, sites of
411 nuclear reverse transcription (Rensen et al.,BioRxiv

412 doi.org/10.1101/2020.04.12.038067). Of note, EdU labeling cannot provide direct
413 information on viral replication, as EdU reduces viral transcription (Rensen et al.,
414 BioRxiv doi.org/10.1101/2020.04.12.038067). Contrariwise, HIV-1 ANCHOR system
415 allows the detection of late reverse transcripts (Fig.1A) that can generate functional
416 proviral DNA, as we observed in HeLa cells, human lymphocytes, and macrophage-
417 like cells (Fig. 2A,B,C, Fig.S2A,B, Fig. S5). Thus, we combined HIV-1 DNA and HIV-1
418 RNA labeling to detect the nuclear landscape where functional viral forms are located.
419 Interestingly, we observed that HIV-1 induces the remodeling of speckles
420 characterized by the presence of SC35 and SON, in novel MLOs enriched in viral
421 proteins, such as IN and CA, as well in cellular factors, like the paraspeckle member,
422 CPSF6, (Fig. 4B, 5A,B, S8). Of note, we observed that CPSF6 is fully included inside
423 organelles marked by SC35 during viral infection, otherwise CPSF6 is usually
424 randomly dispersed in the host nucleus of uninfected cells (Fig.4B, Fig.5A). Likely,
425 CPSF6 relocates to speckles prompted by the virus to usurp functions linked to these
426 MLOs. We show that the formation of CPSF6 cluster is independent of viral DNA
427 integration or viral DNA synthesis (Fig. S8B, S9) and that late reverse transcribed
428 products are excluded from them (Fig. 5A). It is possible that once the vRNA is fully
429 retrotranscribed in vDNA (Rensen et al., BioRxiv doi.org/10.1101/2020.04.12.038067),
430 it will quickly leave the HIV-1 MLOs. In fact, vDNAs were located on average at a
431 distance of ~ 600 nm from the boundary of SC35 (Fig. 5A). MCP-labelled vRNA foci
432 shows that actively transcribing proviruses are also located outside HIV-1 MLOs,
433 indicating that those are not sites of viral integration and replication (Fig.5B,C). We
434 also observed that CPSF6 relocates to the vicinity of LEDGF during HIV infection (Fig.
435 6A). LEDGF is required for the targeting of highly-spliced transcriptional units through
436 its direct interaction with several splicing factors (Singh et al., 2015). LEDGF is also
437 known to determine HIV-1 integration sites distribution in active chromatin sites (Ciuffi
438 et al., 2005; Ferris et al., 2010; Shun et al., 2007), however the visualization of a
439 complex formed by the transcribing provirus and LEDGF remained utopian for a long
440 time. Here, we were able to visualize by fluorescence microscopy a complex formed
441 by the vDNA or vRNA and LEDGF. More than 80% of those complexes are formed by
442 LEDGF uncoupled to CPSF6 (Fig.6B). However, we observed that less than 20% of
443 them can include CPSF6 associated to LEDGF, suggesting that few proviruses need
444 to be near to HIV-1 MLOs without co-localizing, against the majority of them that can
445 replicate farther (Fig. 6B). Whether there is a functional difference between these two

446 complexes it can be further investigated in future studies. Overall, our study highlights
447 the proviral location in the nuclear landscape remodelled by the viral infection (Fig.7).
448 We found that HIV-1-MLOs retain the viral IN but exclude the late retrotranscribed
449 DNA, indicating a role for HIV-1 MLOs prior to the integration step. In particular, we
450 found foci of viral transcription outside HIV-1 MLOs, located in the nuclear periphery,
451 in complex with LEDGF, indicating that HIV-1 MLOs cannot be sites of viral integration
452 and viral transcription. Furthermore, the visualization of the formation of an actively
453 transcribing provirus in complex of a pre-existing cluster of LEDGF, which is known to
454 lay on active chromatin sites (Ciuffi et al., 2005; Singh et al., 2015) (Fig.7), indicates
455 that LEDGF is not part of the viral complex during nuclear entry (Fig.6A). Taken
456 together our results show that functional proviruses establish near to the NE, excluded
457 from CPSF6 clusters, but locate in a favorable transcriptional environment ~ 600nm
458 from HIV-1 MLOs (Fig.7). Our study supports how new single-cell level approaches
459 are pivotal in the study of functional viral nuclear dynamics, to discriminate the cells
460 susceptible to fuel viremia or that concur to viral persistence.

461

462 **Materials and Methods**

463 **Cell lines, primary cells and generation of genetically modified single-cell** 464 **clones.**

465 HEK 293T cells (ATCC) are human embryonic kidney cells used to produce lentiviral
466 vectors and HIV-1 viruses. HeLa P4R5 reporter cells are HeLa CD4⁺ CCR5⁺ CXCR4⁺,
467 carrying the LacZ gene under the control of the HIV-1 LTR promoter (Charneau et al.,
468 1994). HeLa MCP-GFP is a HeLa clone stably expressing MCP-GFP bacterial fusion
469 protein (kind gift from E. Bertrand) (Tantale et al., 2016). Jurkat cells (ATCC) are
470 human immortalized T lymphocytes derived from acute T cells leukemia. THP-1 cells
471 (ATCC) are human immortalized monocyte cells derived from acute monocytic
472 leukemia, which, once seeded, were differentiated into macrophage-like cells under
473 Phorbol 12-myristate 13-acetate (PMA) treatment (167nM). HEK293T cells and HeLa
474 cells were cultivated in DMEM medium supplemented with 10% Fetal Bovine Serum
475 (FBS) and 1% penicillin-streptomycin. Jurkat and THP-1 cells were cultivated in RPMI
476 1640 medium supplemented with 10 % Fetal Bovine Serum (FBS) and 1% penicillin-
477 streptomycin. Primary CD4⁺T cells were purified from healthy donors' blood obtained

478 via EFS (Etablissement Français du Sang, Paris), through density gradient
479 centrifugation with Ficoll 400 and isolation of PBMCs buffy coat. Subsequent positive
480 selection was performed with human CD4⁺ Microbeads (Miltenyi Biotec #130-045-
481 101). The day after CD4⁺ T cells were activated with T cell Activation/Expansion kit
482 (Miltenyi Biotec #130-091-441) according to protocol, complete RPMI 1640 medium
483 was supplemented with interleukin-2 (IL-2) (400 IU/mL). HIV-1 ANCH3 single-cell
484 clone was generated starting from HeLa P4R5 cells which were infected with HIV-1
485 ANCH3 virus at MOI 1. Cells were diluted to 1 cell per well, in 96-well plates. Cell-clone
486 colonies were tested for β -galactosidase expression to check viral expression (kit
487 Roche #11758241001). Positive clones were transduced with lentiviral vector (LV)
488 CMV-OR-GFP for imaging analyses of HIV-1 ANCH3 provirus. All cells were kept in
489 incubator at 37°C and 5% CO₂.

490

491 **Plasmids and lentiviral vector productions**

492 HIV-1 Δ EnvIN_{HA} Δ Nef plasmid encodes for the Δ EnvHIV-1 LAI (BRU) viral genome
493 where the Integrase (IN) protein is fused to the hemagglutinin (HA) tag (kindly provided
494 by F. Mammano)(Petit et al., 1999; Petit et al., 2000). HIV-1 Δ EnvIN_{HA} Δ Nef ANCH3
495 (HIV-1 ANCH3) was obtained through ANCH3 insertion: ANCH3 sequence was cloned
496 by PCR using the template plasmid pANCH3 as we previously described in Blanco et
497 al. (Blanco-Rodriguez et al., 2020). The ANCHORTM technology and sequences are
498 exclusive property of NeoVirTech (Germier et al., 2017; Mariame et al., 2018). HIV-
499 1 Δ Env IN_{HA}(D116A) Δ Nef ANCH3 was obtained by insertional mutagenesis using the
500 QuikChange II XL Site-Directed Mutagenesis kit (Agilent #200522), for integrase (IN)
501 mutation at D116. The lentiviral vector plasmids CMV OR-GFP and CMV OR-
502 SANTAKA were obtained by cloning OR-GFP/OR-SANTAKA (plasmids from
503 NeoVirTech) in a pTripCMV (Δ U3) plasmid through PCR and cloned using restriction
504 site, AgeI and SgrDI. HIV-1 Δ EnvIN_{HA} Δ Nef ANCH3 MS2 plasmid was obtained by
505 inserting the MS2x64 sequence amplified by PCR from pMK123-MS2x64 (Tantale et
506 al., 2016) in HIV-1 Δ EnvIN_{HA} Δ Nef ANCH3. Lentiviral vectors and HIV-1 viruses were
507 produced by transient transfection of HEK293T cells through calcium chloride
508 coprecipitation. Co-transfection was performed as following, LV carrying OR-GFP/OR-
509 SANTAKA: 10 μ g of OR-GFP/OR-SANTAKA LV, 10 μ g of NDK plasmid (gag-pol-tat-
510 rev) and 2.5 μ g of pHCMV-VSV-G envelope expression plasmid; LV carrying MCP-
511 GFP (kindly provided by E. Bertand): 10 μ g of MCP-GFP, 10 μ g of NDK plasmid (gag-

512 pol-tat-rev) and 2.5 µg of pHCMV-VSV-G envelope expression plasmid; HIV-1ΔEnv
513 pseudotyped with VSV-G: 10 µg HIV-1ΔEnvIN_{HA}ΔNef (IN WT or IN(D116A)) plasmid
514 and 2.5 µg of pHCMV-VSV-G plasmid. VSV-G/HIV-1ΔEnv IN_{HA}(D116A) ΔNef ANCH3
515 has been also produced in combination with GIR (Gag-IN-Ruby plasmid, kindly
516 provided by E. Campbell) (Dharan et al., 2016; Hulme et al., 2015). For THP-1
517 infection, HIV-1 ANCH3 or HIV-1 ANCH3 MS2 viruses and LV OR-GFP or LV MCP-
518 GFP were all produced adding 3 µg of SIV_{MAC} Vpx (Durand et al., 2013) at the moment
519 of HEK293T transfection. After the collection of the supernatant 48h post-transfection,
520 lentiviral particles were concentrated by ultracentrifugation for 1 h at 22000 rpm at 4°C
521 and stored at -80°C. Lentiviral vectors and viruses were tittered through late reverse
522 transcripts (LTR) qPCR in HEK293T cells or HeLa P4R5 cells.

523

524 **Quantitative PCR and primers**

525 Total DNA of infected cells was extracted at 6 hours post infection for Late Reverse
526 Transcripts (LRTs) qPCR and 24 hours post infection for 2LTR-circles and ALU-PCR,
527 through QIAmp DNA micro kit (QIAGEN #56304). Real-Time PCR of LRTs was
528 performed to assess DNA synthesis and used to normalize qPCR data on viral input.
529 The reactions were carried on in 20 µL, in iTaqUniversal SYBR Green Supermix (Bio-
530 Rad #1725124) using primers for U3 sequence: U3 FX: 5'-
531 TTCCGCTGGGGACTTTCCAGGG-3', U3 RX: 5'-AGGCTCAGATCTGGTCTAACC-3'.
532 Real-Time PCR of 2LTR-circles was used to assess nuclear import efficiency.
533 Reactions were performed in 20 µL, in Maxima Probe/ROX qPCR Mastermix
534 (ThermoFisher #K0232) using primers for 2LTR-circle junction: 2LTR FX: 5'-
535 AACTAGGGAACCCACTGCTTAAG-3', 2LTR RX: 5'-
536 TCCACAGATCAAGGATATCTTGTC-3', 2-LTR probe: 5'-(FAM)-
537 ACACTACTTGAAGCACTCAAG-GCAAGCTTT-(TAMRA)-3'. Real-Time PCR of
538 proviral integrations consisting in a first non-kinetic PCR in 50 µL in Platinum SuperFi
539 DNA Polymerase (ThermoFisher #12351250) and in a second step of qPCR reaction
540 in 20 µL in Maxima Probe/ROX qPCR Mastermix (ThermoFisher #K0232). Primer Alu
541 166: 5'-TCCCAGCTACTCGGGAGGCTGAGG-3', Alu 2: 5'-
542 GCCTCCCAAAGTGCTGGGATTACAG-3', LambdaU3: 5'-
543 ATGCCACGTAAGCGAACTTTCCGCTGGGGACTTTCCAGGG-3' for ALU PCR;
544 Lambda: 5'-ATGCCACGTAAGCGAACT-3', U5: 5'-CTGACTAAAAGGGTCTGAGG-
545 3', Probe: 5'-(FAM)- TTAAGCCTCAATAAAGCTTGCCTTGAGTGC-(TAMRA) for ALU

546 qPCR(Di Nunzio et al., 2013). In all experiments β -actin detection was used for
547 normalization. β -actin FX: 5'-AACACCCCAGCCATGTACGT-3', β -actin RX: 5-
548 CGGTGAGGATCTTCATGAGGTAGT-3', β -actin probe: (FAM)-
549 CCAGCCAGGTCCAGACGCAGGA-(BHQ1).

550

551 **Epifluorescence and Immunofluorescence**

552 For imaging studies in adherent cell-lines, the cells were plated on coverslips (12mm
553 diameter in 24 well plates or 18 mm diameter in 12 well plate, #1, ThermoFisher) at
554 least 24h before fixation. For HIV-1 ANCH3 imaging, HeLa P4R5 cells were
555 transduced with OR-GFP LV (MOI 0.2) and then infected within the following 3 days
556 with HIV-1 ANCH3 or HIV-1 IN_{D116A} ANCH3 at different MOIs. NEVIRAPINE (NEV) 10
557 μ M or RALTEGRAVIR (RAL) 20 μ M were used to block respectively DNA synthesis
558 and integration. For HIV-1 MS2 RNA imaging HeLa-MCP cells were infected with HIV
559 ANCH3 MS2 and fixed 24h post infection. For co-visualization of vDNA and vRNA,
560 HeLa MCP-GFP cells were transduced with OR-SANTAKA LV (MOI 1) and 24h later
561 infected with HIV-1 ANCH3 MS2. All THP-1 cells studies were carried on after 48h of
562 differentiation with PMA (167 nM), then, they were transduced with LV OR-GFP Vpx
563 (MOI 5) or LV MCP-GFP Vpx (MOI 20) and 48 h later they were infected with HIV-1
564 ANCH3 Vpx (30 h or 3 days p.i) or HIV-1 ANCH3 MS2 \pm Vpx (3 days p.i.). The medium
565 was always supplemented with PMA. For HIV-1 ANCH3 imaging in primary
566 lymphocytes, CD4⁺T cells, 3 days after activation, were transduced with OR-GFP LV
567 (MOI 0.5) for 72 h and then infected with HIV-1 ANCH3 for 20 h. The day of fixation
568 cells in suspension were seeded on poly-L-lysine (Sigma #P4707) coated coverslips
569 and centrifuged to allow attachment. The day of fixation all cells were washed with
570 PBS and fixed with 4% PFA for 15 minutes. For protein staining, cells were treated
571 with glycine 0.15% for 10 min, permeabilized with Triton X-100 0.5% for 30 min and
572 blocked with 1% bovine serum albumin (BSA) for 30 min. All incubations were carried
573 out at room temperature, in the dark, in humid chamber, 1h with primary antibodies
574 and 45 min with secondary antibodies. Washes between antibody incubations and
575 antibodies dilution were done in 1% BSA. Primary antibodies were diluted as follows:
576 anti-HA 1:500 (Roche #11867423001), anti-HIV-1 p24 (CA) 1:400 (NIH #3537), anti-
577 CPSF6 1:400 (Novus Biologicals #NBP1-85676), anti-SC35 1:200 (Abcam #ab11826),
578 anti-LEDGF 1:200 (BD Bioscience #611715). Secondary antibodies used were the

579 following: Goat anti-Mouse Alexa-647 1:300 (1:100 for CA) (Invitrogen #A21235);
580 Donkey anti-Rat Alexa-488 1:100 (Invitrogen A21208) for IN-HA) or Goat anti-Rat
581 Alexa-647 1:300 (Invitrogen #A21247) (1:100 for IN-HA); Donkey anti-Rabbit Cy3
582 1:500 (Jackson Lab #711-165-152). Finally, cells were stained with Hoechst 33342
583 1:5000 (Invitrogen #H3570) for 5 minutes. Coverslips were mounted on glass slides
584 (Star Frost) with Prolong Diamond Antifade Mountant (Life Technologies #P36970).
585 Confocal microscopy was carried out with a Zeiss LSM700 inverted microscope, with
586 a 63X objective (Plan Aplanachromat, oil immersion, NA=1.4).

587

588 **RNA FISH**

589 For RNA FISH studies in adherent cell-lines, the cells were seeded on coverslips. HeLa
590 P4R5 cells expressing OR-GFP were infected with HIV-1 ANCH3 (MOI 30) with or
591 without RAL 20 μ M and fixed 24h post infection or infected with HIV-1 ANCH3 (MOI 5)
592 for 48h or 7 days. Jurkat T cells expressing OR-GFP were infected with HIV-1 ANCH3
593 (MOI 5) and fixed 48h post infection. Primary activated CD4⁺T cells, 3 days after
594 activation, were together transduced with OR-GFP LV (MOI 10) and infected with HIV-
595 1 ANCH3 (MOI 5) for 3 days. THP-1 cells were differentiated with PMA for 48h and,
596 after 48h transduction with LV OR-GFP Vpx (MOI 5), the cells were infected with HIV-
597 1 ANCH3 Vpx (MOI 20) for 3 days. The day of fixation Jurkat and CD4⁺T cells were
598 seeded on poly-L-lysine (Sigma #P4707) coated coverslips and centrifuged to allow
599 attachment. The day of fixation all cells were washed with PBS and fixed with 4% PFA
600 for 15 minutes and incubated in 70% ethanol at -20°C at least for one night. Primary
601 smiFISH probes have a targeting sequence against HIV-1 *pol* transcript and a shared
602 readout sequence for secondary probe alignment. Twenty-four smiFISH probes (table
603 I) against HIV *pol* were designed with Oligostan (Tsanov et al., 2016) and purchased
604 from Integrated DNA Technologies (IDT). Primary probes were pre-hybridized with a
605 secondary FLAP probe conjugated to Cy5 fluorophore through pairing with the readout
606 sequence. Washes and hybridization were performed with Stellaris Buffers (WASH
607 buffer A, WASH buffer B, Hybridization Buffer; LGC Biosearch Technologies),
608 following the manufacturer protocol. Hybridization with the probe was carried out at
609 37°C in a dark humid chamber for 5 hours. Finally, cells were stained with Hoechst
610 33342 1:5000 (Invitrogen #H3570) for 5 minutes. Coverslips were mounted on glass
611 slides (Star Frost) with Prolong Diamond Antifade Mountant (Life Technologies

612 #P36970). Confocal microscopy was carried out with a Zeiss inverted LSM700
613 microscope, with a 63X objective (Plan Apochromat, oil immersion, NA=1.4).

614 **Time-lapse microscopy**

615 For all live imaging studies, the cells were plated on a polymer-coverslip bottom μ -Dish
616 35 mm (ibidi #81156) or in μ -Dish 35 mm Quad (10,000 cells per chamber, ibidi
617 #80416) and 2D or 3D videos were acquired with an UltraView VOX Spinning Disk
618 Microscope (Perkin-Elmer), based on a CSU-X spinning-disk (Yokogawa), and using
619 a 63X objective (Plan Apochromat, oil immersion, NA=1.4). For integrated provirus
620 imaging, HIV-1 ANCH3 single-cell clone was transduced with OR-GFP LV (MOI 0.2)
621 for 3 days and then plated on the polymer-bottom dish (10^5 cells) the day before
622 acquisition. On the other hand, HeLa P4R5 cells were infected with HIV-1 ANCH3 (MOI
623 10) and five days post infection cells were plated on the polymer-coverslip bottom dish
624 and transduced with LV OR-GFP for 24h. For non-integrated viral DNA, HeLa P4R5
625 were firstly transduced with OR-GFP LV (MOI 0.2) and the day after cells were plated
626 on polymer-bottom dish and infected with HIV-1 ANCH3 (MOI 10) with RAL 20 μ M or
627 with the mutated HIV-1 IN_{HA} (D116A) ANCH3. Cells were imaged in continue for 10
628 minutes. Experiments of co-live tracking of IN-Ruby (GIR) and vDNA labeled by OR-
629 GFP were performed in HeLa P4R5 cells transduced with LV OR-GFP (MOI 0.2). The
630 following day, cells were infected with HIV-1 IN_{HA}(D116A) ANCH3 complemented with
631 the GIR plasmid using an MOI 50. Fluorescence images were taken every 30 seconds
632 for up to 9h post infection. For co-live-imaging of vDNA and vRNA foci, HeLa MCP-
633 GFP cells were transduced with OR-SANTAKA LV (MOI 1) and 24 h later infected with
634 HIV-1 ANCH3 MS2 (MOI 50).

635

636 **Imaging Analysis and Statistics**

637 All data were analyzed in GraphPad Prism 8 (GraphPad Software, La Jolla California
638 USA, www.graphpad.com), computing Student's t test for 2 comparisons, One-way
639 ANOVA for more than two comparisons and Pearson's correlation. P values symbols:
640 ns = non significant, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$. All
641 images and videos were analyzed in Fiji (Schindelin et al., 2012) and Icy version
642 2.0.3.0 (de Chaumont et al., 2012) software.

643 All images and video were processed and analyzed in Fiji (Schindelin et al., 2012) or
644 in Icy software version 2.0.3.0 (de Chaumont et al., 2012). Here below the custom-
645 made protocols:

646 *Particle tracking and MSD measurement*

647 3D videos were analyzed in Fiji (Schindelin et al., 2012) and viral nuclear particles
648 were tracked along time in 3D using TrackMate plugin (Tinevez et al., 2017). The
649 resulting tracks were exported in MATLAB (The MathWorks, Natick, USA) and then
650 used for mean-squared displacement (MSD) analysis with @msdanalyzer (Tarantino
651 et al., 2014). MSD curves were fitted by a straight line from which we derived the value
652 of the diffusion coefficient: $D = a / (2 * nDim)$, where « a » is the slope of the MSD curve
653 and « nDim » the dimensionality of the image. MSD curves for which the R^2 value was
654 lower than 0.8 were discarded from analysis. Only the initial 25% of the MSD curves
655 were used for the linear fit.

656 *vDNA-vRNA association analysis*

657 For the analysis of RNA FISH images ± RAL in HeLa P4R5 cells, 2D confocal images
658 were analysed with Icy software version 2.0.3.0 (de Chaumont et al., 2012) using a
659 custom made protocol ([http://icy.bioimageanalysis.org/protocol/batch-spots-
660 detections-and-statistics](http://icy.bioimageanalysis.org/protocol/batch-spots-detections-and-statistics) and doi: 10.5281/zenodo.3925262, example of HeLa
661 confocal image available at doi: 10.5281/zenodo.3925213). vDNA spots were detected
662 extracting the green channel and applying first a median filter of half size = 1 and then
663 3, the Spot Detector with a scale of 1 (sensitivity to 55) on the nuclear ROI only. Data
664 from the spot detection (e.g. mean fluorescence intensity) were exported for analysis.
665 vRNA mean fluorescence intensity signal was computed from the nuclei ROI. For
666 vDNA - vRNA association studies in Jurkat cells, 3D confocal images were analyzed
667 in Icy version 2.0.3.0 using two successive custom made protocols (first protocol:
668 [http://icy.bioimageanalysis.org/protocol/nuclei-segmentation-from-maximum-
669 intensity-projections/](http://icy.bioimageanalysis.org/protocol/nuclei-segmentation-from-maximum-intensity-projections/) with doi: 10.5281/zenodo.3925181) and second protocol:
670 [http://icy.bioimageanalysis.org/protocol/spots-detection-and-colocalisation-analysis-
671 with-soda/](http://icy.bioimageanalysis.org/protocol/spots-detection-and-colocalisation-analysis-with-soda/) with doi: 10.5281/zenodo.3925191, example of Jurkat confocal image
672 available at doi: 10.5281/zenodo.3925213). Nuclei were segmented on a maximum
673 intensity projection of the Hoechst channel using the Icy block Gaussian filter with a
674 radius of sigma x=5, y=5 and z=5 pixels, the Thresholder block with a manual threshold
675 of 5000 and the Label Extractor block, which uses connected components to separate
676 the nuclei. Cy5 spots were detected with the HK Means block (Dufour et al.,

677 doi.org/10.1109/ICPR.2008.4761748).The logical operation block was used to keep
678 only the spots detected in the nucleus. These spots were added as input parameter
679 “List detection 1” of the SODA block. EGFP spots were detected using the log3D block
680 to enhance the spots, inverting the log transformed image using the Math Operation
681 Expression block with the following expression: $A*B$ with A scalar=-1 and B the log
682 transformed image, detecting the spots with the Local Extrema block and segmenting
683 the spots with a dilation and a connected components operations. The logical operation
684 block was used to keep only the spots detected in the nucleus. These spots were
685 added as input parameter “List detection 2” of the SODA block. The nucleus ROI was
686 kept as input parameter for “ROI of Analysis (Cell’s shape)”. The Cy5 and GFP spots
687 ROIs were then imported in the SODA block (Lagache et al., 2018) to analyse
688 colocalization of spots within the nucleus only with a maximum radius of 10, a step of
689 1 and no fixed search distance.

690 *vDNA/vRNA nuclear envelope distance analysis*

691 For the analysis of HeLa P4R5 cells 48 h p.i. and 7 days p.i. and in THP-1 3 days p.i.
692 3D RNA FISH images, the nuclei were segmented on the Hoechst channel using
693 background subtraction followed by a multilevel Otsu thresholding. The resulting nuclei
694 masks were then directly applied on the vDNA and vRNA channels to select the
695 relevant 3D information inside each labelled region. Inside these regions, vRNA spots
696 were identified using 3D non-local means denoising (Buades et al., "Non-local means
697 denoising." *Image Processing On Line* 1 (2011): 208-212) and automated
698 segmentation, while vDNA spots were extracted with the image analysis pipeline
699 previously described (Komatsu et al., 2018). For each vDNA and vRNA spot in was
700 computed the distance to the nuclear envelope in the 2D z-plane corresponding to the
701 spot centroid location.

702 *SC35-vDNA distance analysis*

703 The 3D confocal images were processed for multi-channel image splitting, vDNA and
704 SC35 segmentation. The automated 3D segmentation of cell nuclei included a
705 preliminary non-local means denoising step to cope with the strong signal
706 heterogeneity and the segmentation of SC35 was readjusted to low contrast
707 conditions. The 3D boundary-to-boundary distance (rather than centroid-to-centroid
708 which was done for vDNA/vRNA-NE analysis) between each vDNA spot and its closest
709 SC35 speckle was computed.

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724

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FIGURE LEGENDS

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951 **Figure 1. Detection and live tracking of nuclear HIV-1 DNA forms. A)** Scheme of
952 vDNA labeled by the HIV-1 ANCHOR system. ANCH3 is the target sequence of the
953 OR protein. ANCH3 has been cloned in the HIV-1 genome in place of Nef gene (HIV-
954 1 ANCH3). The late retrotranscribed DNA of HIV-1 ANCH3 can be visualized in cells
955 stably expressing the OR-GFP protein, thanks to the accumulation of OR protein to the
956 double-stranded ANCH3 sequence. Viral DNA detection occurs mostly in the nucleus,
957 where late reverse transcripts are abundant and are more likely to be exposed to the
958 binding of OR-GFP. Starting from early time points of infection, it is possible to follow
959 HIV-1 ANCH3 in target cells. The labelling results in the appearance of a bright
960 fluorescent spot that can be tracked over time (time post-infection is indicated in the
961 upper-left corner). The time-lapse images are representative of three independent
962 experiments. The cartoon model was created with BioRender.com. **B)** Confocal
963 images of HeLa P4R5 cells expressing OR-GFP infected at MOI 30 with HIV-1 ANCH3,
964 HIV-1 ANCH3 + RAL (20 μ M), HIV-1 ANCH3 IN_{D116A} and HIV-1 ANCH3 + NEV
965 (10 μ M), 24 h p.i. On the right, histogram plots of 2-LTR circles and ALU-PCR,
966 comparing the different infection conditions, represent the nuclear import and
967 integration rates, respectively. All graphs contain \pm SD. **C)** Confocal image of HIV-1
968 ANCH3 provirus in a single-cell clone. The clone was transduced with OR-GFP LV for
969 the detection of HIV-1 integrated DNA. On the right, histogram plot of ALU-PCR and
970 β -galactosidase expression of the HIV-1 ANCH3 single-cell clone compared to non-
971 infected cells (NI). All graphs contain \pm SD. **C)** On the left, Tukey's box plots of the
972 diffusion coefficient (D (nm^2/s)) of episomal and integrated HIV-1 DNA forms tracked
973 in 3D time lapses (movies S1, S2, S3). On the right, an example of live track for each
974 experimental group is shown. Low D corresponds to D (nm^2/s) < 5 and high D to $5 < D$
975 (nm^2/s) < 20 . The color range indicates the time up to 10 min. Statistics: Student's t
976 test for two comparisons, one-way ANOVA for more than two comparisons. ns=non-
977 significant, *= $p \leq 0.05$, **= $p \leq 0.01$, ***= $p \leq 0.001$, ****= $p \leq 0.0001$. Scale bars: 5 μ m.
978

979 **Figure 2. HIV-1 transcriptional activity in dividing and non-dividing cells. A)**
980 Labeling of vDNA and vRNA in HeLa cells expressing OR-GFP obtained by coupling
981 of HIV-1 ANCHOR (vDNA) with RNA FISH (vRNA). On the left, confocal images of

982 HIV-1 RNA FISH with or without RAL (20 μ M), 24 h p.i. On the right, scatter plot with
983 bars showing the mean fluorescence intensity of the vRNA signal (red) per nucleus
984 and of vDNA spots detection (green) (for vRNA: n = 13 cells (-RAL), 16 cells (+RAL);
985 for vDNA: 95 (-RAL) and 141 (+RAL) spots, n = 13 cells (-RAL), 16 cells (+RAL)). The
986 analysis was performed in 2D. **B)** Labeling of vDNA and vRNA in CD4⁺T cells
987 expressing OR-GFP. On the left, confocal image of HIV-1 RNA FISH in primary
988 activated CD4⁺ T cells, 3 days p.i.. On the right, scatter plot with bars showing the
989 percentage of vDNA associated to vRNA signal and vice versa, per nucleus (n= 262
990 (vDNA spots); 64 (vRNA foci) in infected Jurkat cells, 48 h p.i. (some examples of cells
991 are shown in Fig. S5). The analysis was performed in 3D. **C)** The same techniques
992 have been used to label vDNA and vRNA in macrophage-like cells, THP-1, expressing
993 OR-GFP. On the left, confocal image of infected THP-1, 3 days p.i. On the right, scatter
994 plot with bars showing the percentage of vDNA associated to vRNA signal and vice
995 versa, per nucleus (n=130 (vDNA spots), 99 (vRNA foci). The analysis was performed
996 in 3D. All graphs contain \pm SD. Statistics: Student's t test. ns=non-significant, *=p \leq
997 0.05, **=p \leq 0.01, ***=p \leq 0.001, ****=p \leq 0.0001. Scale bars: 5 μ m.

998

999 **Figure 3. Distance of HIV-1 provirus from the nuclear envelope in dividing and**
1000 **non-dividing cells. A)** On the left, confocal images of HIV-1 DNA labeling by HIV-1
1001 ANCHOR system and HIV-1 RNA labeling by RNA FISH in HeLa cells expressing OR-
1002 GFP infected with HIV-1 ANCH3 at 48 h p.i. compared to 7 days p.i.. On the right,
1003 scatter plot with bars indicating the distance from the nuclear envelope (NE) per
1004 detected vDNA spot (green) and vRNA focus (red) (n=vDNA spots: 83 (48 h p.i.) and
1005 16 (7 d p.i.); n=vRNA foci: 52 (48h p.i.) and 18 (7d p.i.)). **B)** On the left, confocal images
1006 to detect vDNA and vRNA in THP-1 cells expressing OR-GFP infected with HIV-1
1007 ANCH3, 3 days p.i.. On the right, scatter plot with bars indicating the distance from the
1008 nuclear envelope (NE) per detected vDNA spot (green) and vRNA focus (red) (n=139
1009 vDNA spots; n= 126 vRNA foci). **C)** Scatter plot with bars comparing the distances
1010 from the nuclear envelope (NE) per detected vRNA focus in HeLa P4R5, 48h p.i. and
1011 THP-1, 3 days p.i. (vRNA foci: 52 (HeLa), 126 (THP-1)). All the analyses were
1012 performed in 3D. All graphs contain \pm SD. Statistics: Student's t test for two
1013 comparisons, one-way ANOVA for more than two comparisons. ns=non-significant,
1014 *=p \leq 0.05, **=p \leq 0.01, ***=p \leq 0.001, ****=p \leq 0.0001. Scale bars: 5 μ m, inset 1 μ m.
1015

1016 **Figure 4. HIV-1 integrase location in dividing and non-dividing cells. A)** Confocal
1017 images of primary activated CD4⁺ T and THP-1 cells expressing OR-GFP infected with
1018 HIV-1 ANCH3, 20h and 30h p.i, respectively, compared to uninfected cells. Co-
1019 visualization of vDNA (green) and viral IN (magenta). On the bottom scatter plot with
1020 bars comparing the percentages of vDNAs associated to IN per nucleus (n=vDNA: 54
1021 (CD4+T), 100 (THP-1)). The analysis was performed in 3D. **B)** Confocal images of
1022 primary activated CD4⁺ T and THP-1 cells infected with HIV-1 ANCH3, 20h and 30h
1023 p.i, respectively, compared to uninfected cells. Co-visualization of CPSF6 (red) and
1024 viral IN (cyan). The graphs show the percentages of IN-CPSF6 association events
1025 and vice versa, per nucleus (n= for CD4+T: 46 (CPSF6), 33 (IN); for THP-1: 206
1026 (CPSF6), 132 (IN)). All graphs contain \pm SD. Statistics: Student's t test. ns=non-
1027 significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Scale bars: 5 μ m.

1028
1029 **Figure 5. HIV-1 proviruses are excluded from CPSF6/SC35 MLOs. A)** Confocal
1030 images of THP-1 cells expressing OR-GFP infected with HIV-1 ANCH3, 3 days p.i., to
1031 co-visualize vDNA (green), CPSF6 (red), SC35 (cyan) or to co-visualize vDNA (green),
1032 SON (red), SC35 (cyan), compared to uninfected cells. On the right, the graphs show:
1033 the percentages of CPSF6 clusters associated to vDNA or to SC35, per nucleus (n=
1034 CPSF6 clusters > 50); the comparison of Pearson's r coefficients of SON-SC35
1035 colocalization between infected and uninfected cells, per nucleus (n = 25 cells (HIV-1
1036 ANCH3), 19 cells (NI)); the average distance of each vDNA from the closest SC35-
1037 speckle border (n= vDNAs: 83). **B)** Confocal images of THP-1 cells expressing MCP-
1038 GFP infected with HIV-1 ANCH3 MS2, 3 days p.i., to co-visualize vRNA (green),
1039 CPSF6 (red), SC35 (cyan), compared to uninfected cells. The graph shows the
1040 percentages of CPSF6 clusters associated to vRNA foci or to SC35 (n= CPSF6
1041 clusters: 42). **C)** Scatter plot with bars indicating the percentage of vDNA or vRNA
1042 associated to CPSF6, per nucleus (n= 69 (vDNA), 30 (vRNA)). All the analyses were
1043 performed in 3D. All graphs contain \pm SD. Statistics: Student's t test. ns=non-
1044 significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Scale bars: 5 μ m,
1045 inset 1 μ m.

1046
1047 **Figure 6. HIV-1 functional proviruses are in complex with LEDGF. A)** Confocal
1048 images of THP-1 cells expressing OR-GFP infected with HIV-1 ANCH3, 3 days p.i., to
1049 co-visualize CPSF6 (red) and LEDGF (white), compared to uninfected cells. On the

1050 left scatter plot of the percentages of CPSF6 found associated to LEDGF clusters per
1051 nucleus; on the right, scatter plot of the LEDGF and CPSF6 count per nucleus (n = 16
1052 cells). Scale bar: 5 μ m, inset 2 μ m. **B)** Confocal images of THP-1 cells expressing OR-
1053 GFP infected with HIV-1 ANCH3 and expressing MCP-GFP infected with HIV-1
1054 ANCH3 MS2, to co-visualize vDNA or vRNA (green), CPSF6 (red) and LEDGF (white).
1055 The scatter plot shows the percentage of vDNA or vRNA associated to LEDGF (n= 33
1056 (vDNA), 15 (vRNA)). The pie charts show the percentages of the 2 populations of
1057 LEDGF clusters associated to vDNA or vRNA: LEDGF alone or LEDGF in proximity of
1058 CPSF6. Scale bar: 5 μ m, inset 1 μ m. All the analyses were performed in 3D. Statistics:
1059 Student's t test. ns=non-significant, *=p \leq 0.05, **=p \leq 0.01, ***=p \leq 0.001, ****=p \leq
1060 0.0001.

1061

1062 **Figure 7. HIV-1 restructures the nuclear landscape for efficient replication.** Once
1063 retrotranscribed and imported in the nucleus, HIV-1 genome integrates in open
1064 chromatin regions enriched in LEDGF proteins, in less than 2 micrometers from the
1065 nuclear envelope. In addition, upon infection, CPSF6 clusters in SC35 nuclear
1066 speckles with the viral IN restructuring the nuclear landscape and inducing HIV-1
1067 MLOs formation. Late retrotranscribed DNA and proviruses are excluded from CPSF6
1068 clusters. HIV-1 transcription is favored by the nuclear environment surrounding proviral
1069 sites that may profit from the proximity to SC35 and other splicing factors for post-
1070 transcriptional processes. Created with BioRender.com.

1071

A

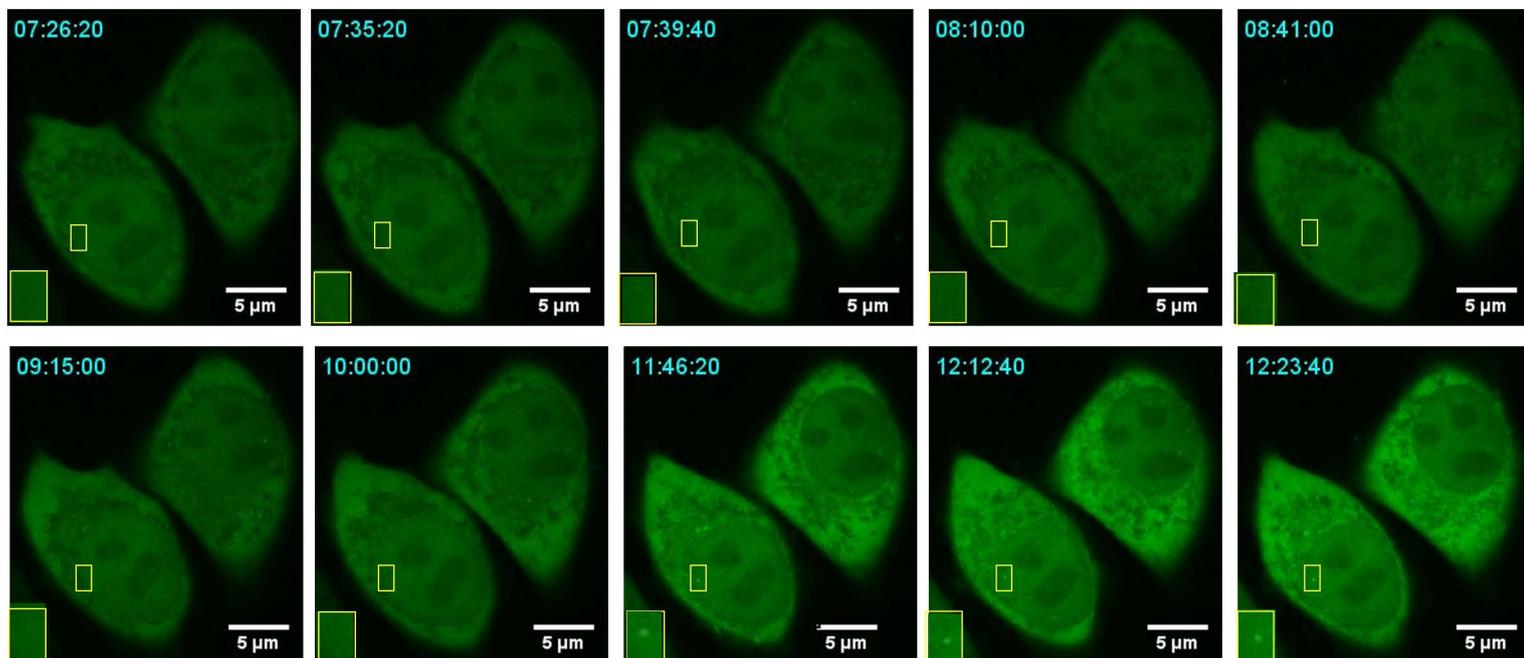
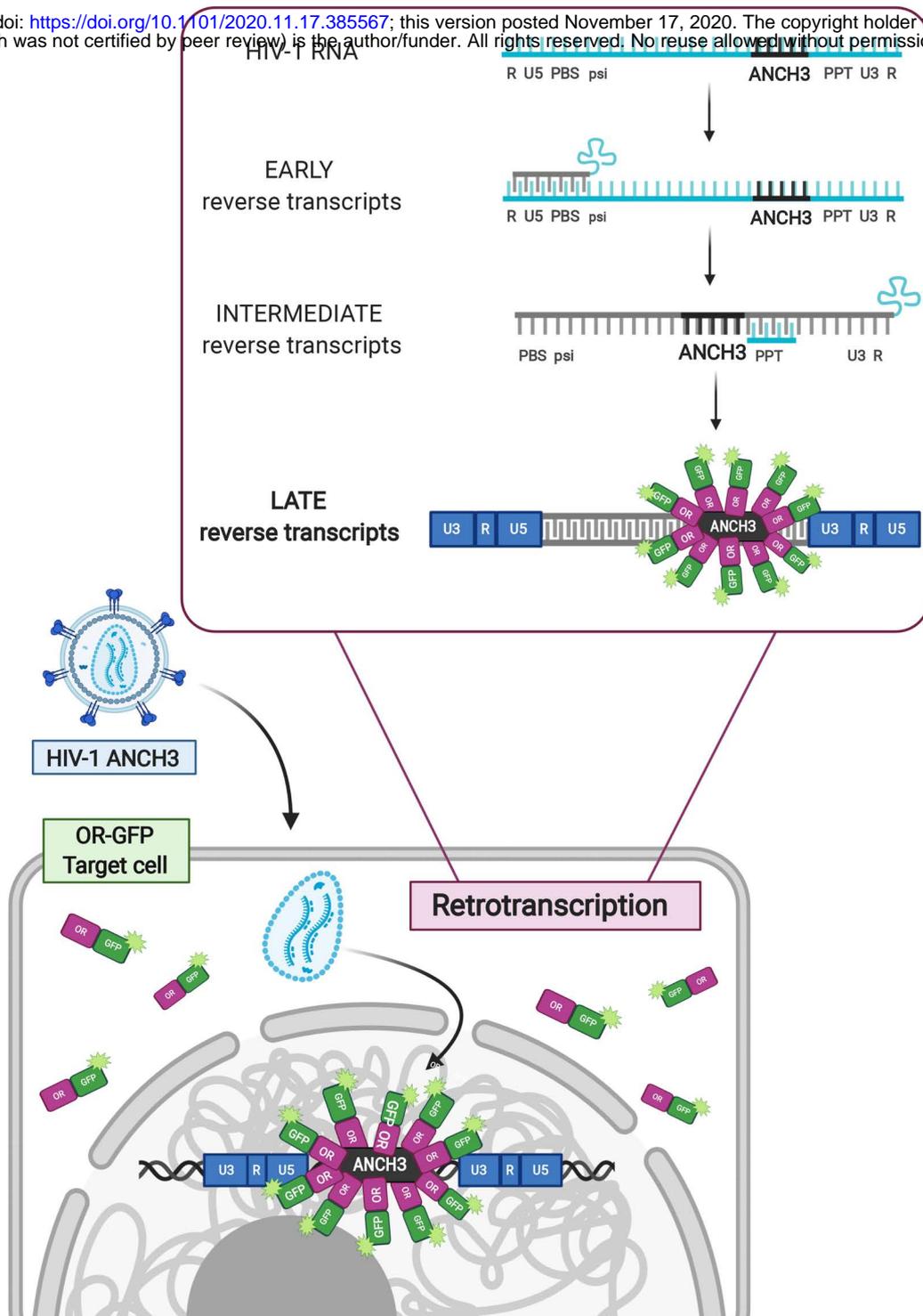
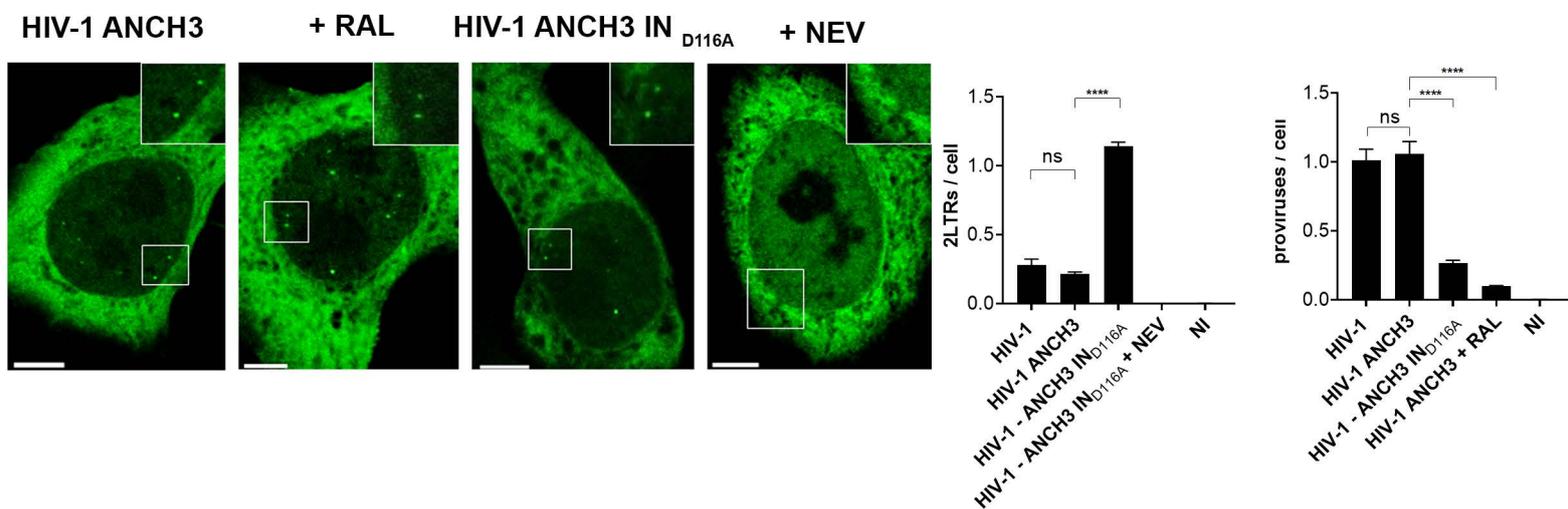
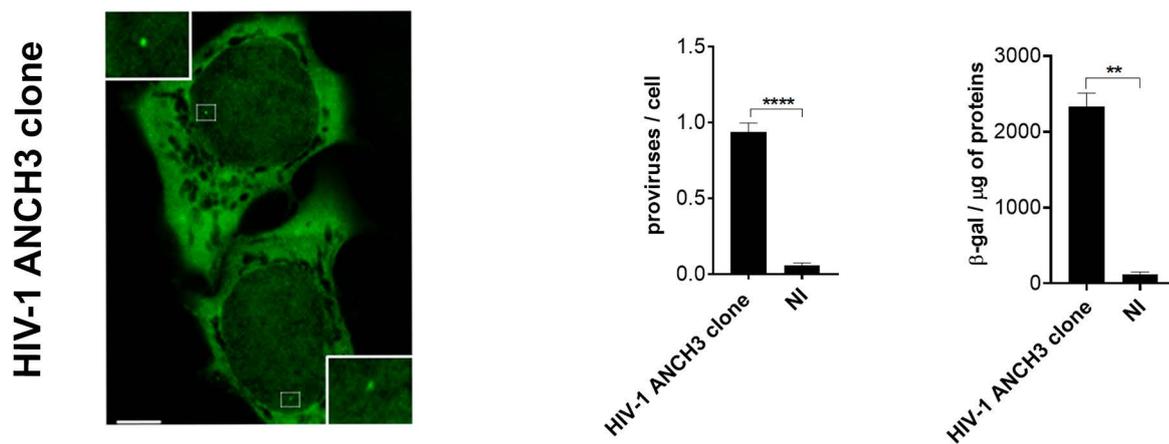


Figure 1

B



C



D

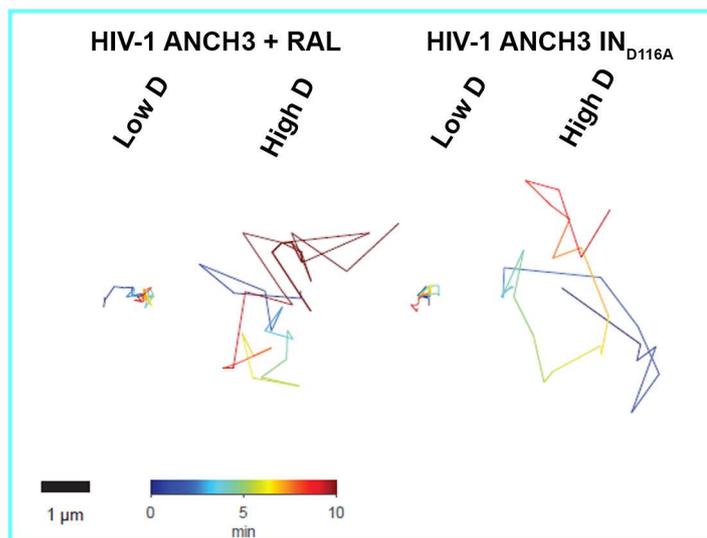
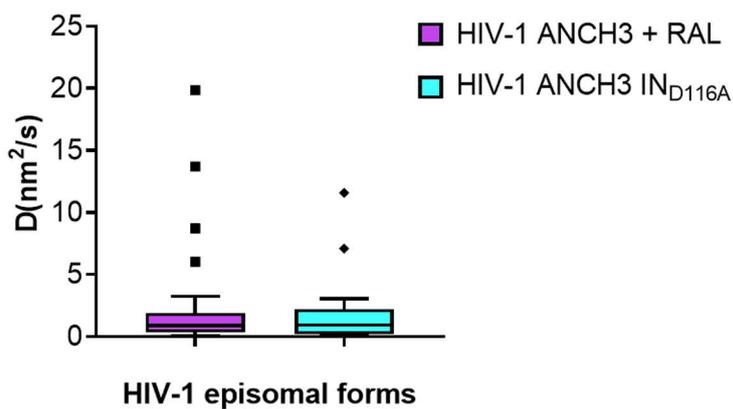
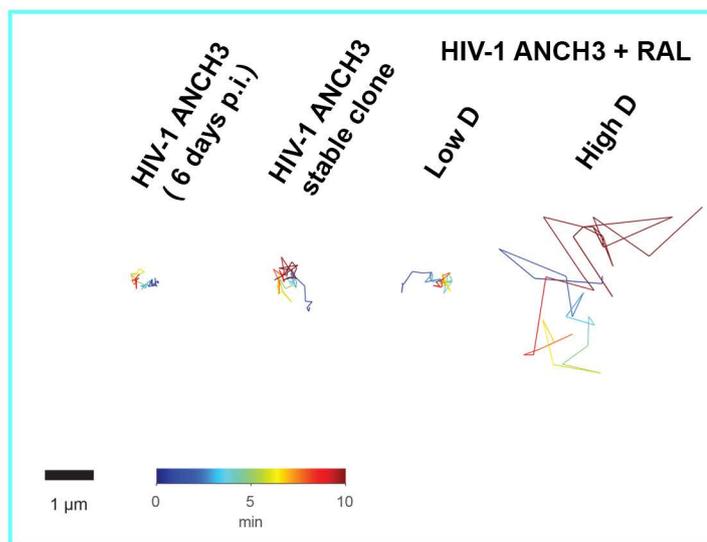
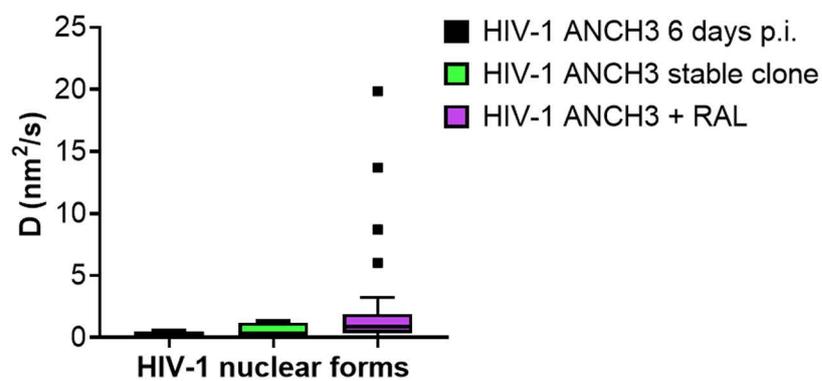


Figure 1

HeLa cells

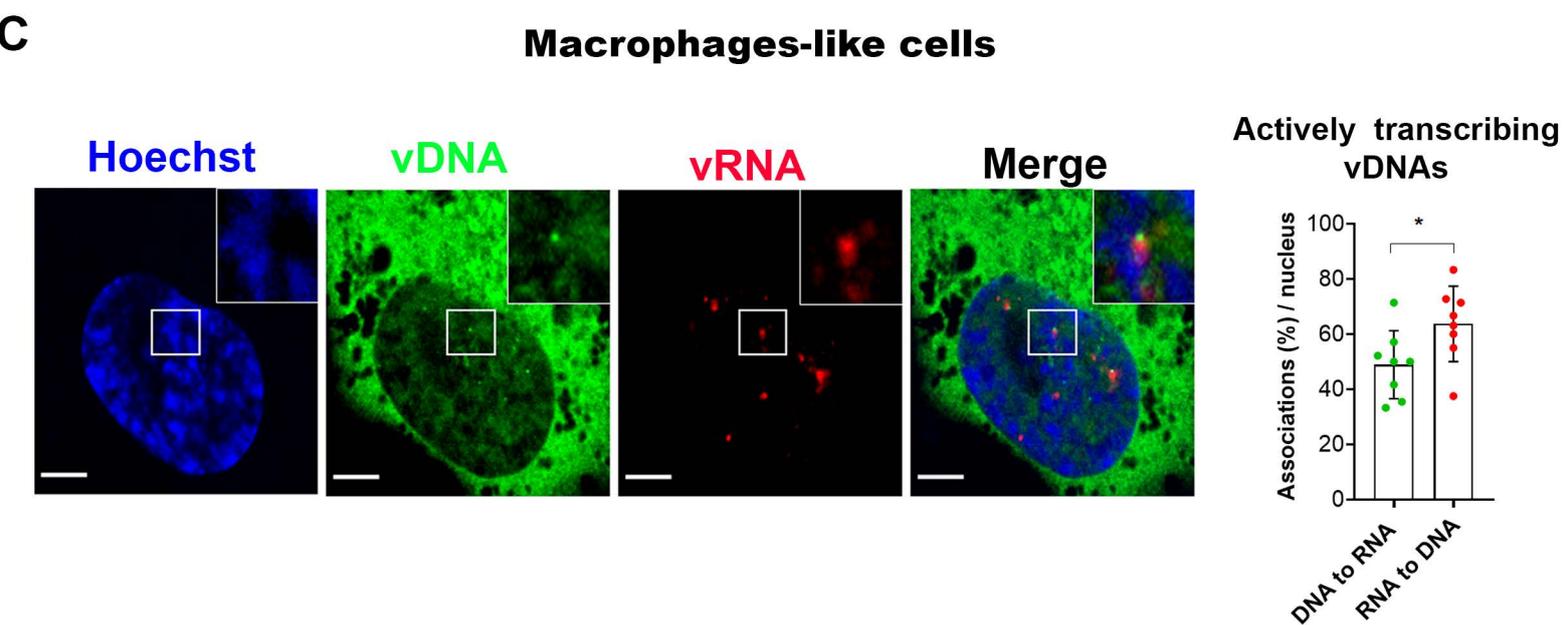
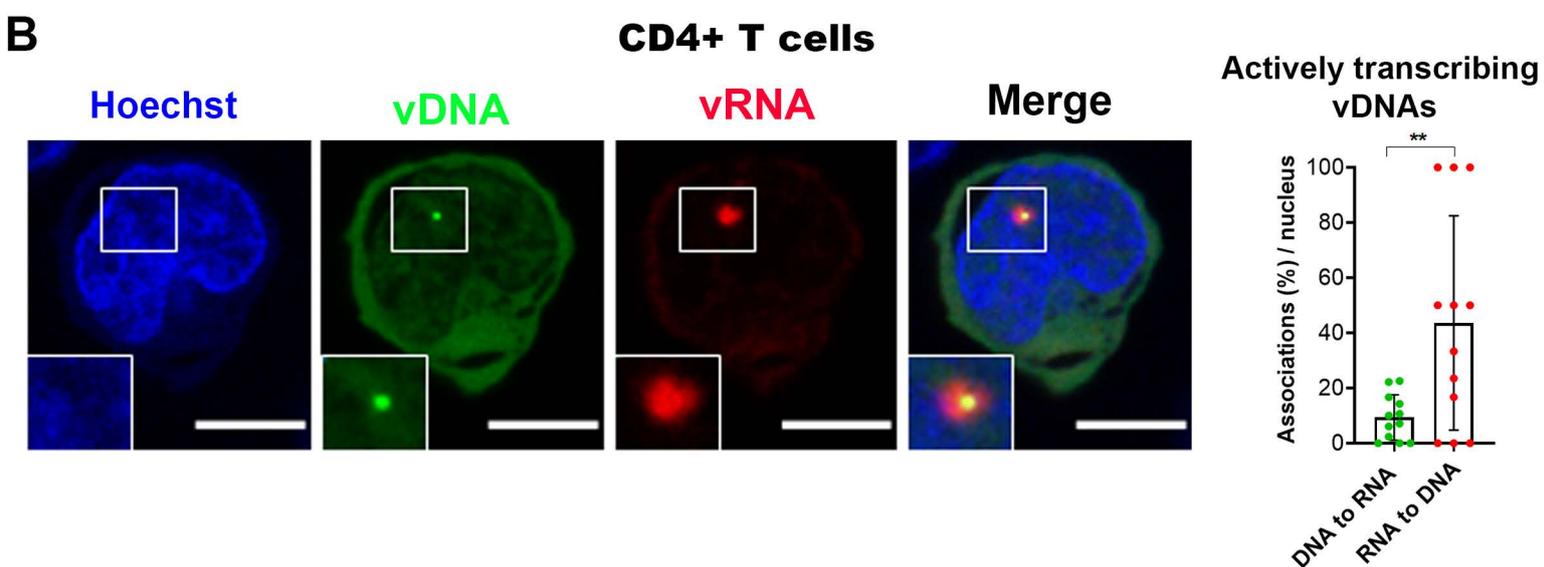
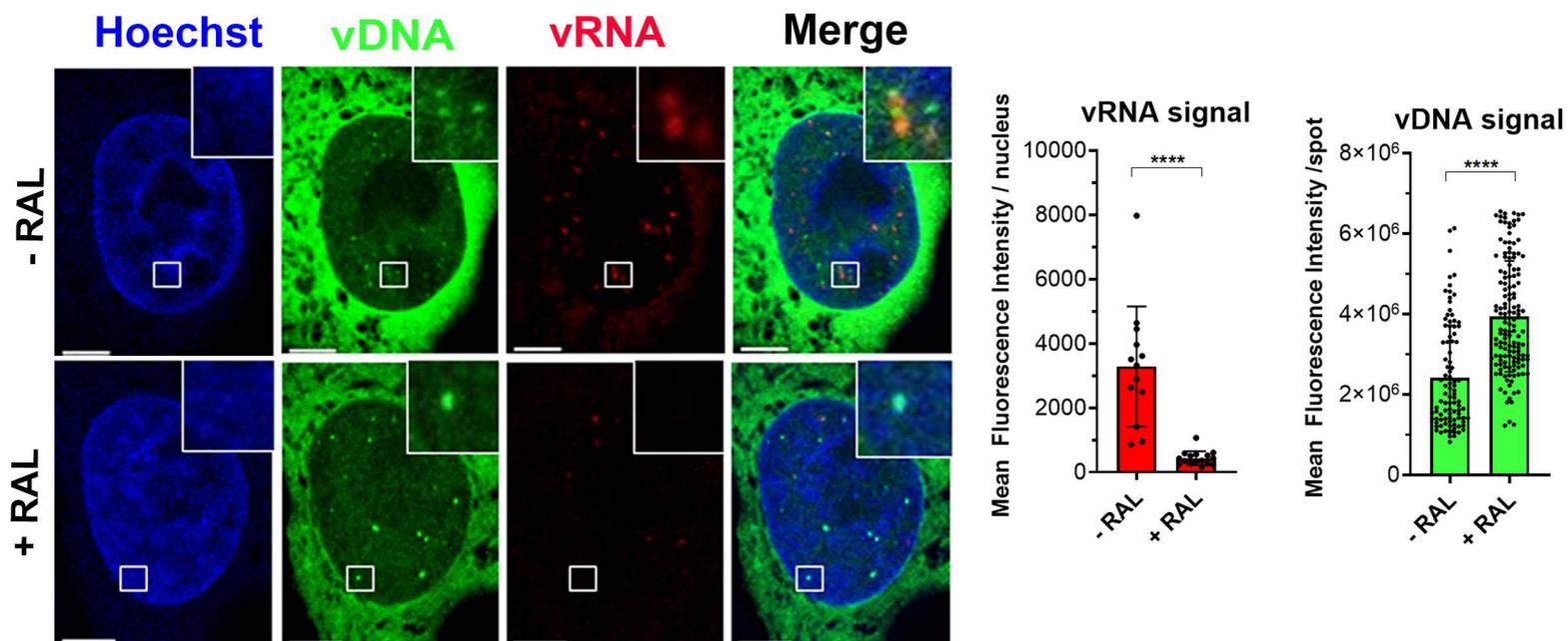
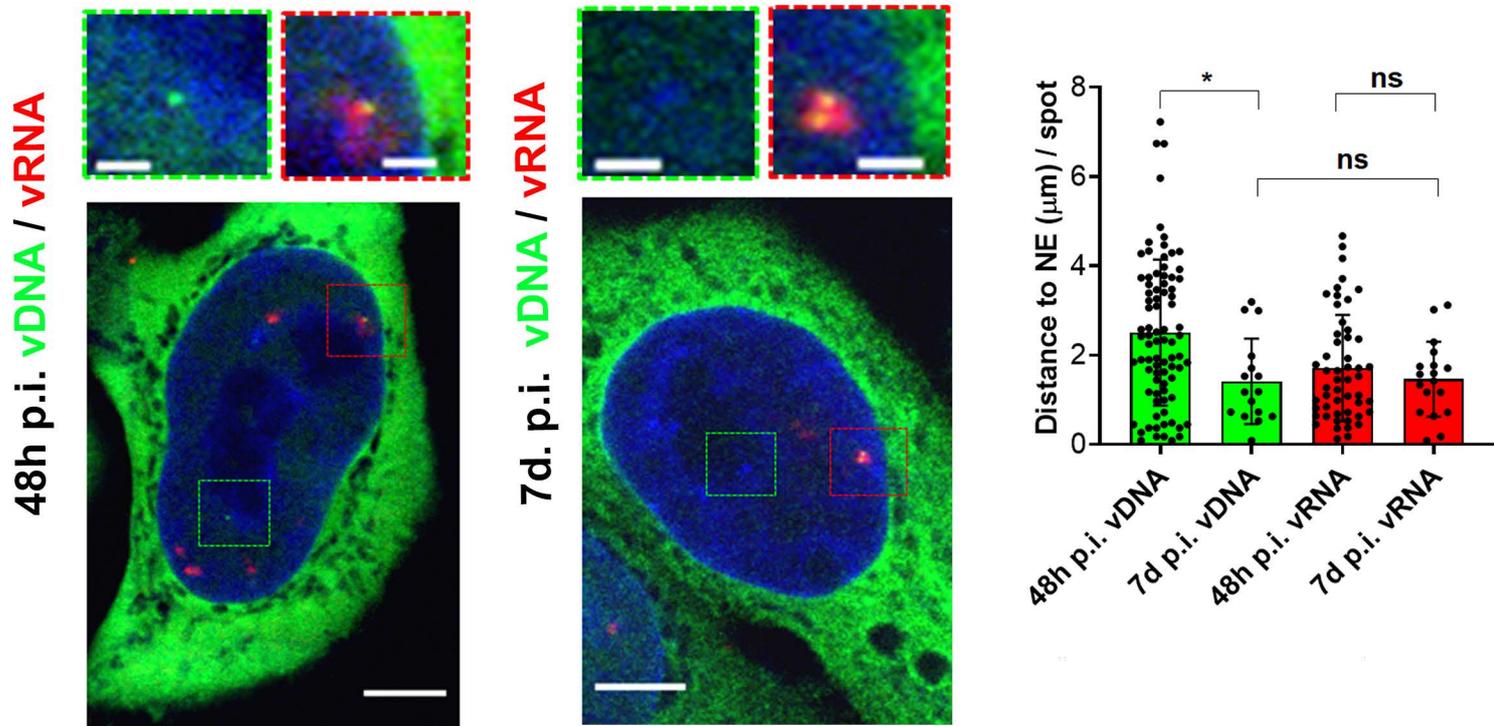


Figure 2

Dividing cells: HeLa



Non-dividing cells: THP-1

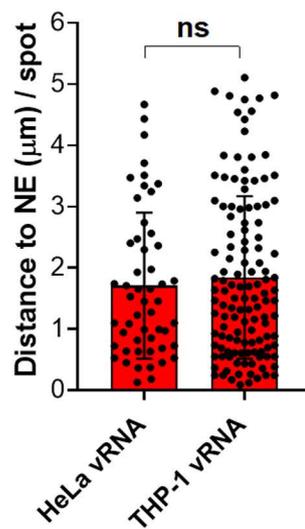
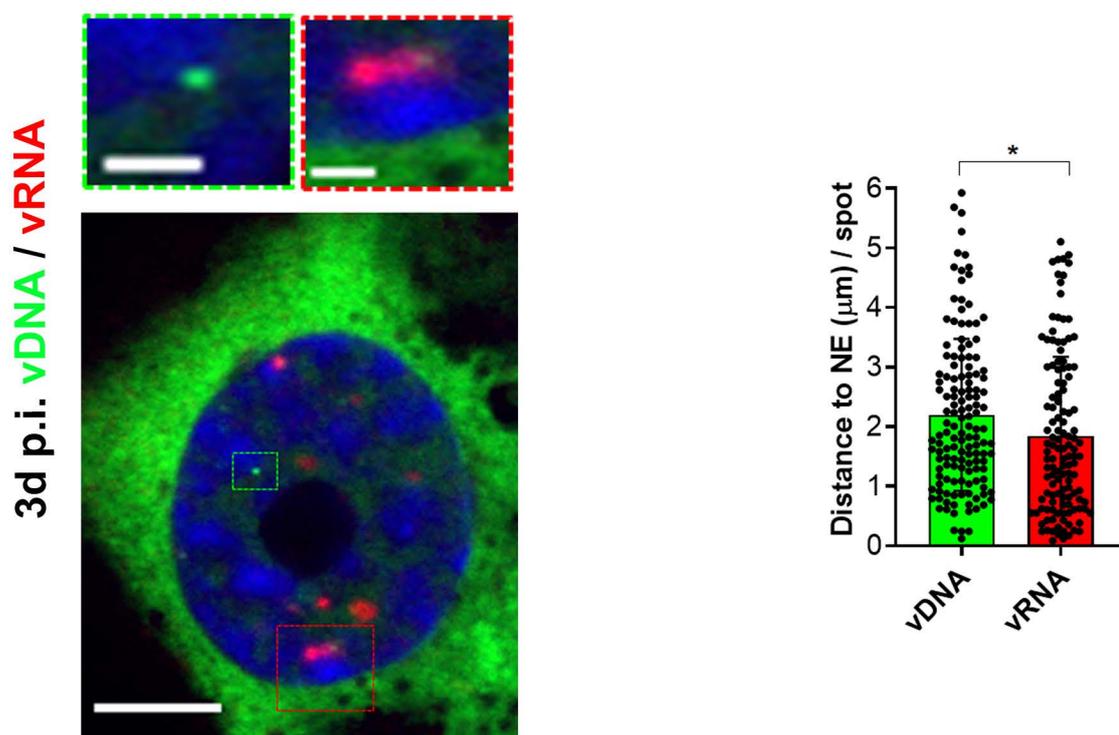


Figure 3

B

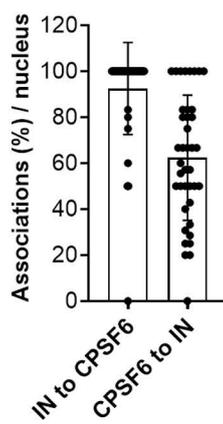
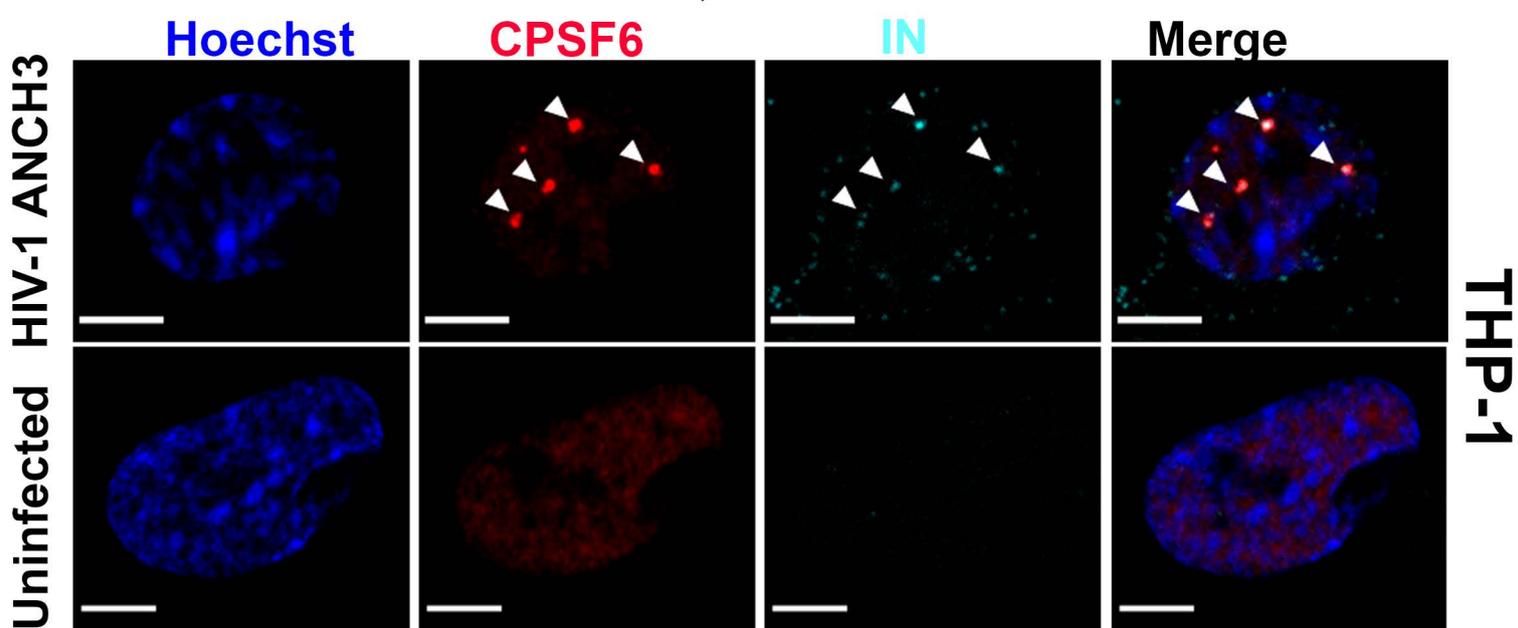
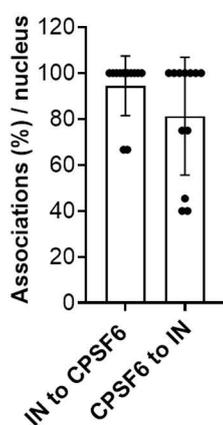
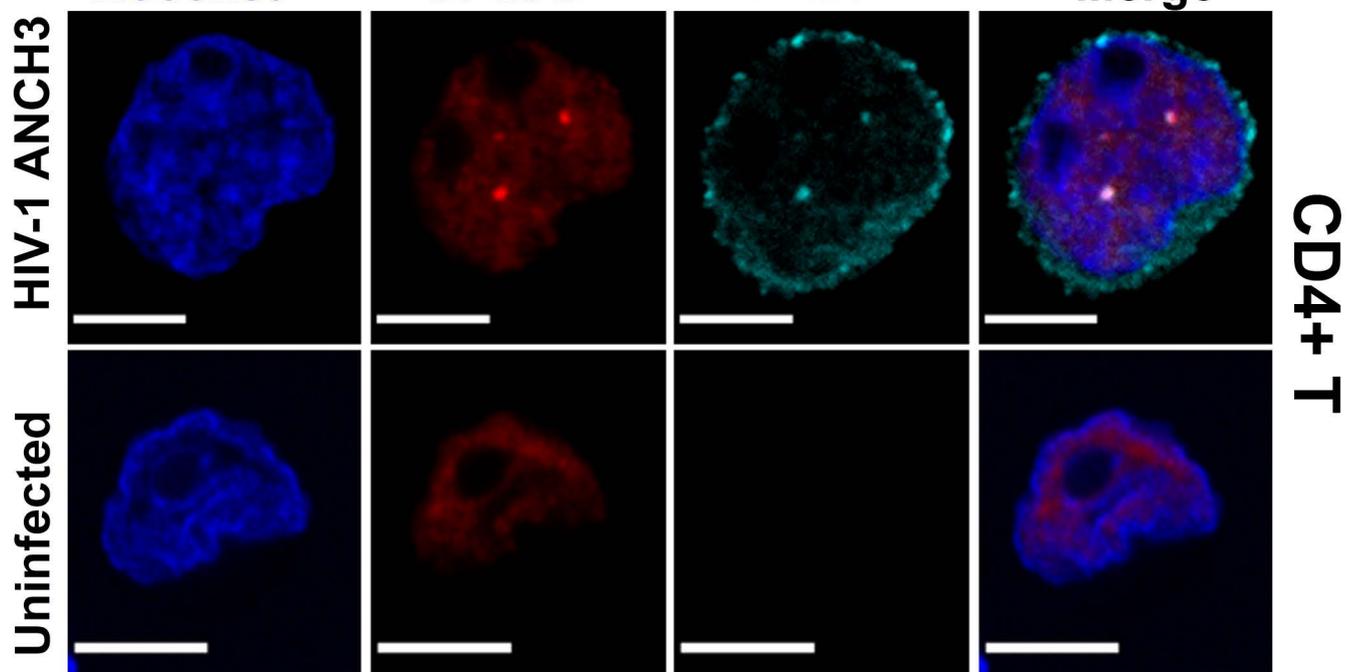
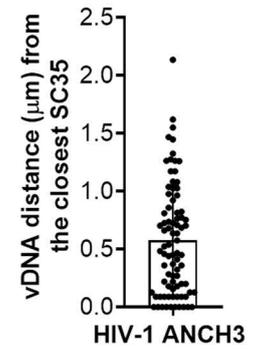
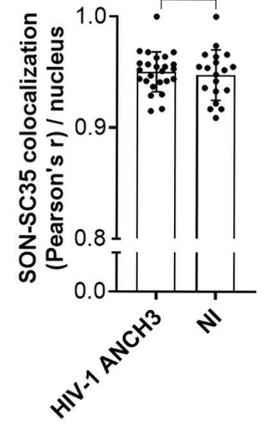
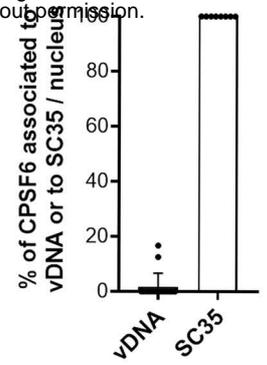
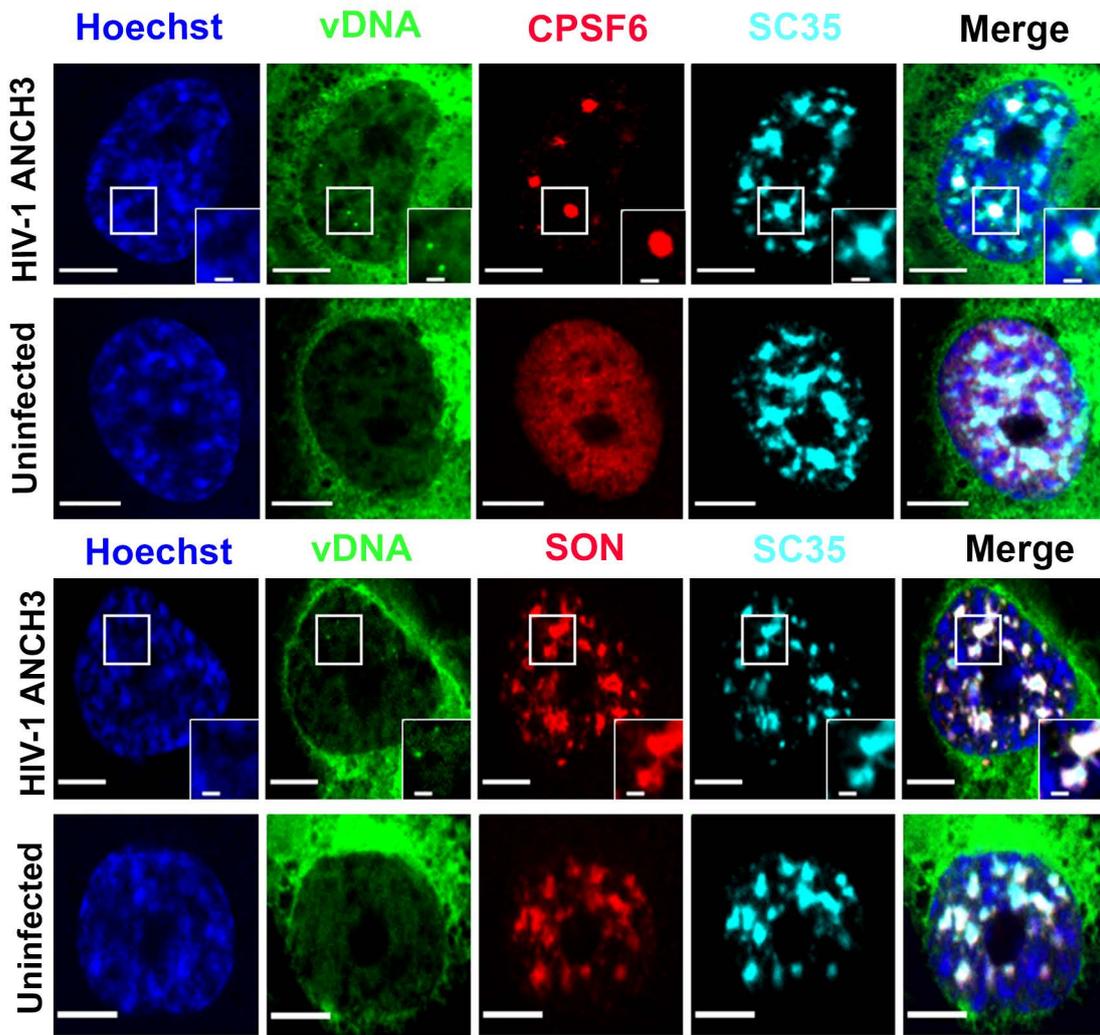


Figure 4

A



B

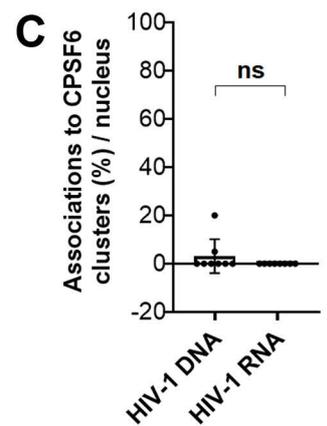
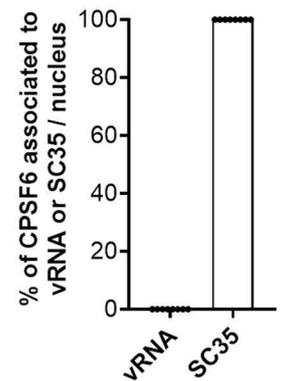
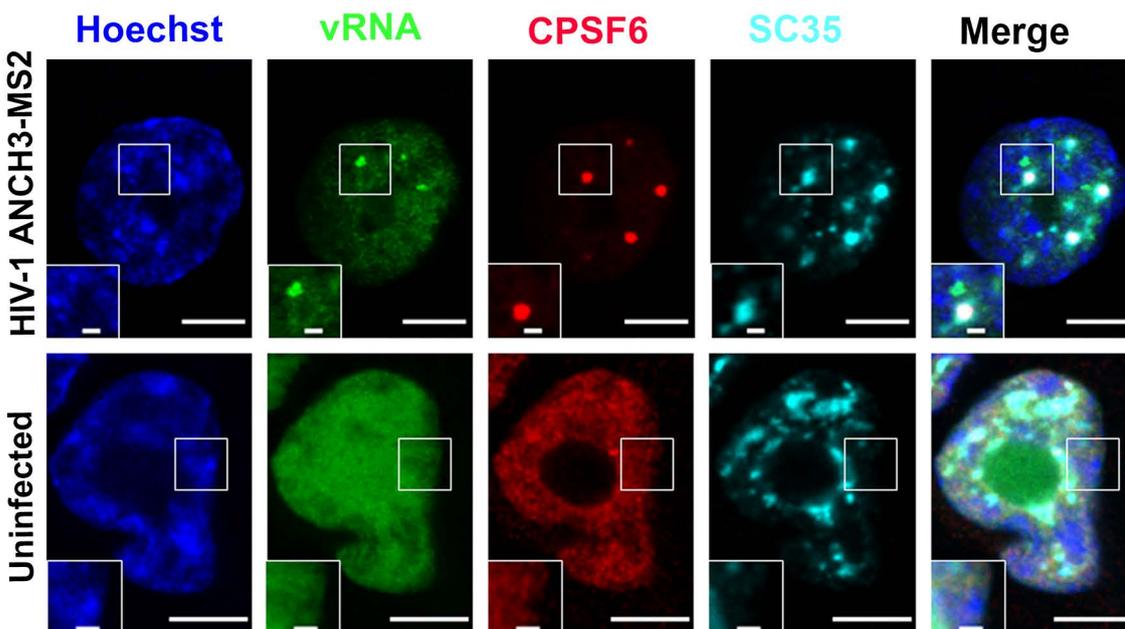
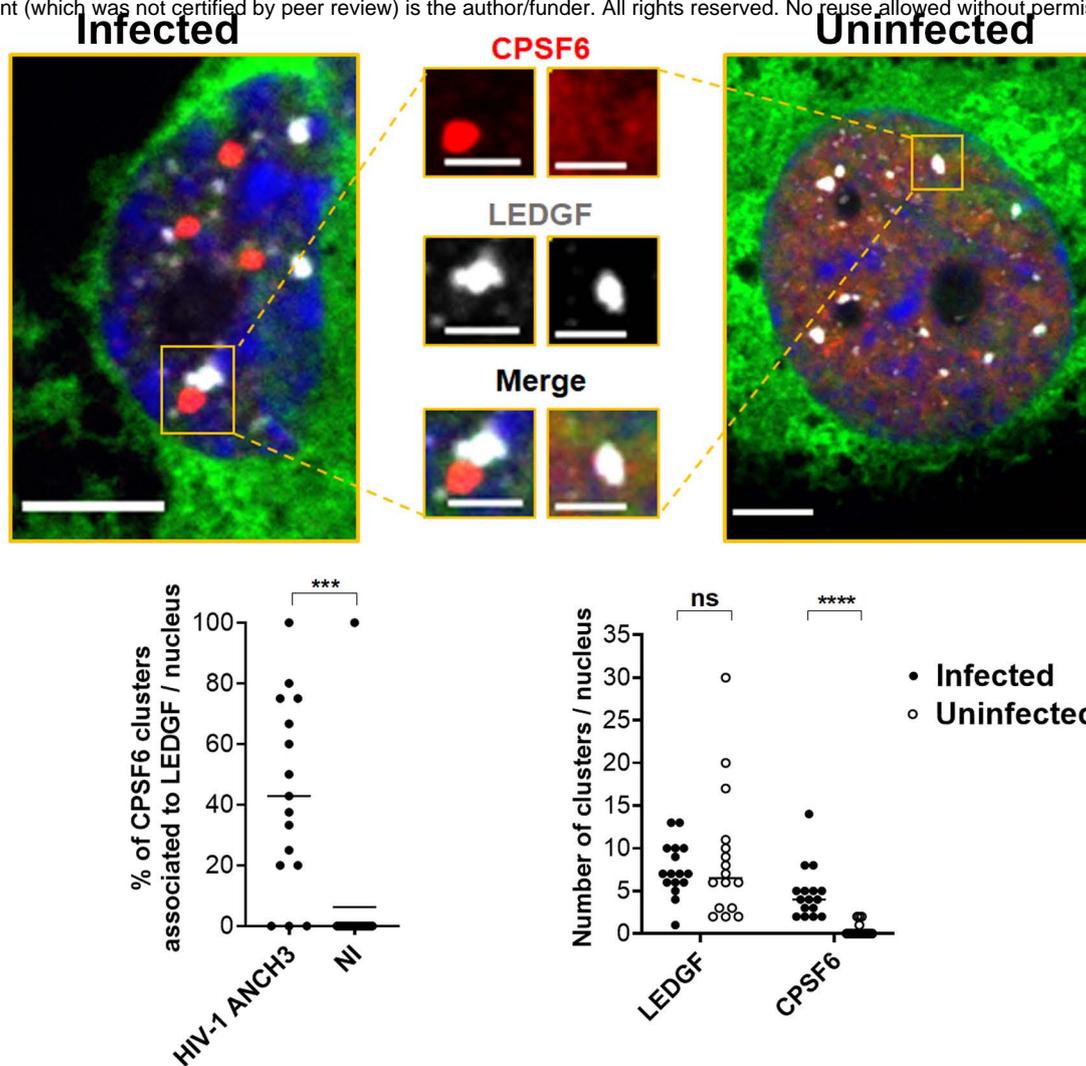


Figure 5

A



B

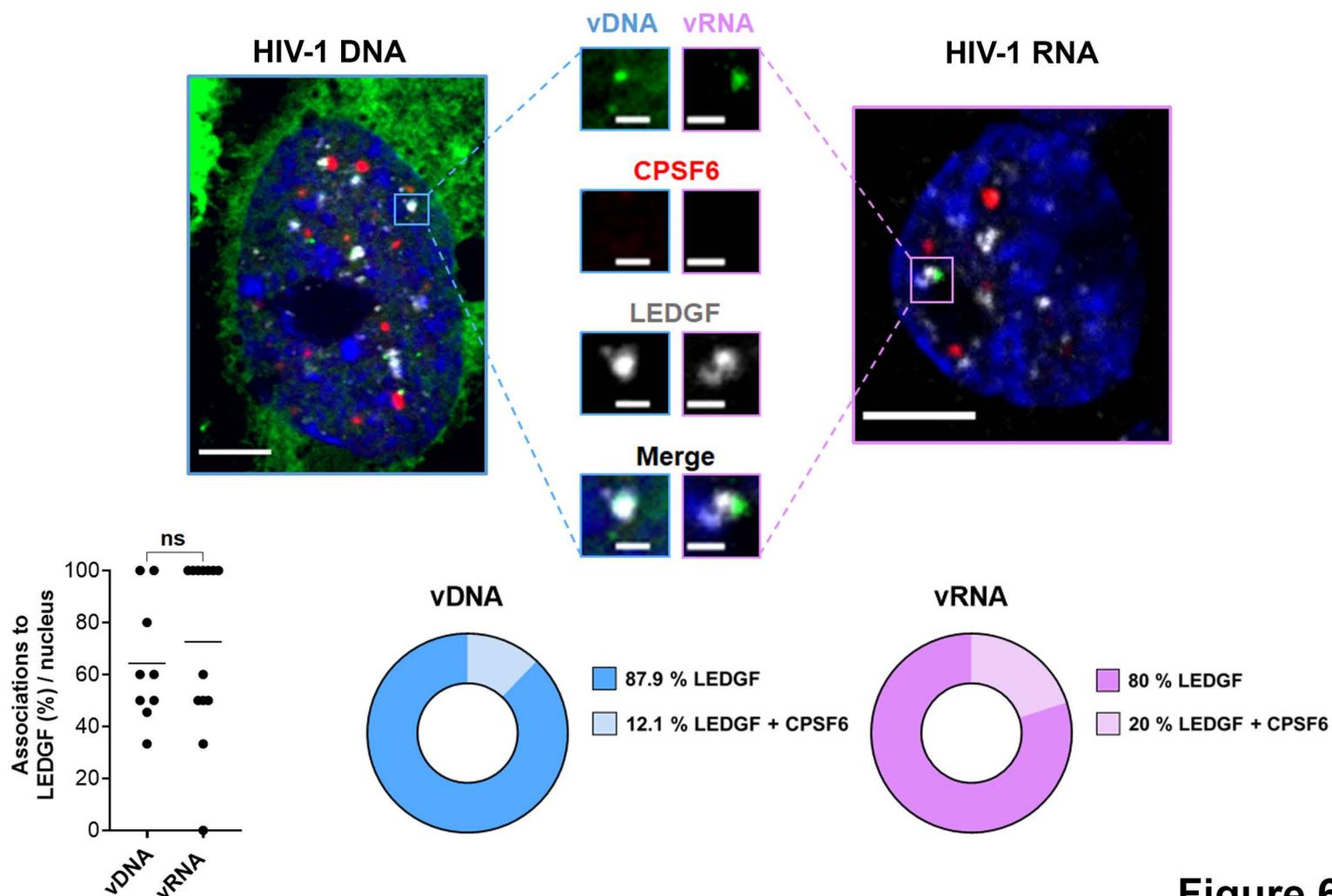


Figure 6

Uninfected

Infected

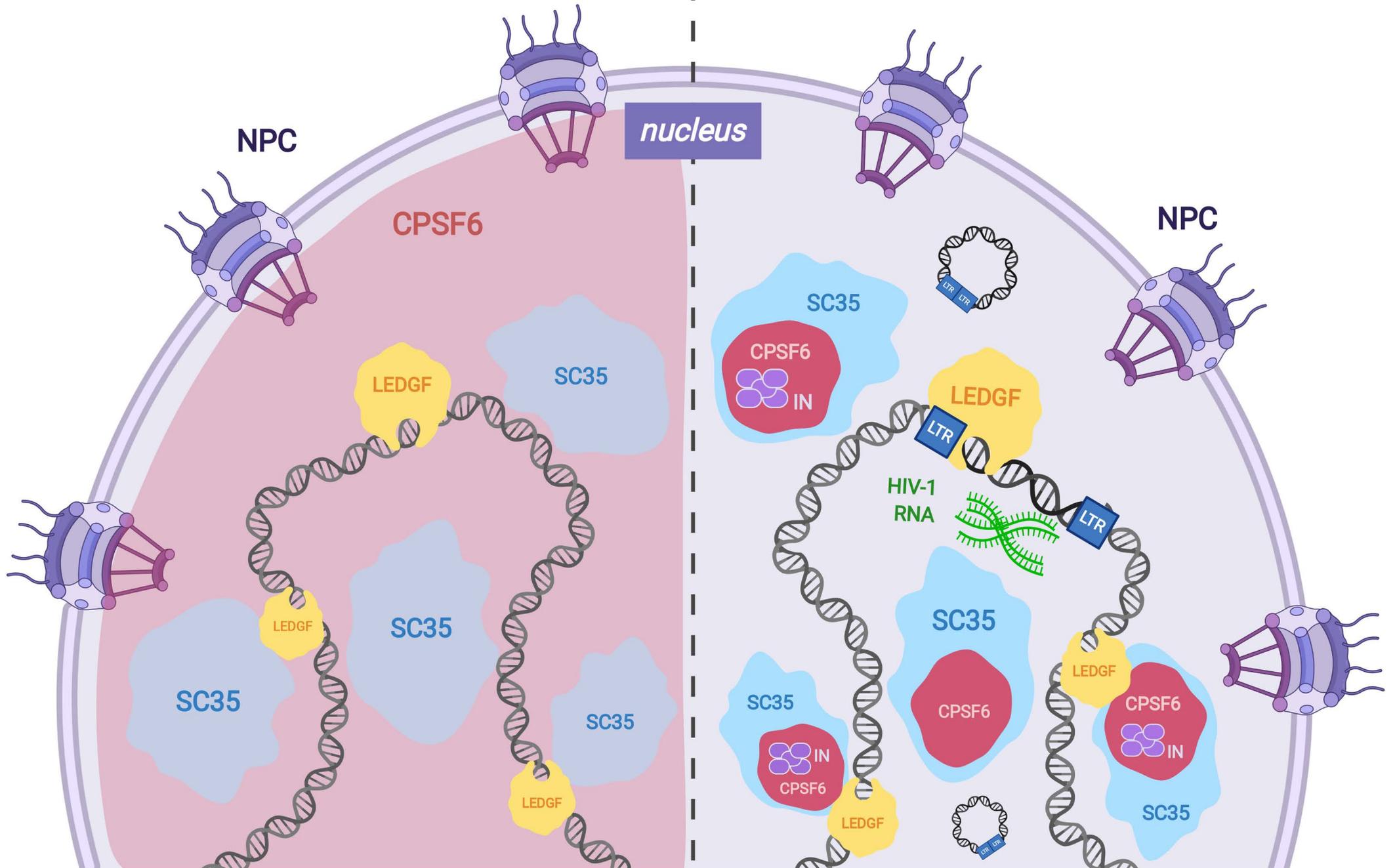


Figure 7