

1 **TRIM5 α restriction of HIV-1-N74D viruses in lymphocytes is**
2 **caused by a loss of cyclophilin A protection**

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21 **ABSTRACT**

22 The core of HIV-1 viruses bearing the capsid change N74D (HIV-1-N74D) do not
23 bind the human protein cleavage and polyadenylation specificity factor subunit 6
24 (CPSF6). In addition, HIV-1-N74D viruses have altered patterns of integration site
25 preference in human cell lines. In primary human CD4⁺ T cells, HIV-1-N74D viruses
26 exhibit infectivity defects when compared to wild type. The reason for this loss of
27 infectivity in primary cells is unknown. We first investigated whether loss of CPSF6
28 binding accounts for the loss of infectivity. Depletion of CPSF6 in human CD4⁺ T cells
29 did not affect the early stages of wild-type HIV-1 replication, suggesting that defective
30 infectivity in the case of HIV-1-N74D is not due to the loss of CPSF6 binding. Based on
31 our previous result that cyclophilin A (Cyp A) protected HIV-1 from human tripartite
32 motif-containing protein 5 α (TRIM5 α_{hu}) restriction in CD4⁺ T cells, we tested whether
33 TRIM5 α_{hu} was involved in the decreased infectivity observed for HIV-1-N74D. Depletion
34 of TRIM5 α_{hu} in CD4⁺ T cells rescued the infectivity of HIV-1-N74D, suggesting that HIV-
35 1-N74D cores interacted with TRIM5 α_{hu} . Accordingly, TRIM5 α_{hu} binding to HIV-1-N74D
36 cores was increased compared with that of wild-type cores, and consistently, HIV-1-
37 N74D cores lost their ability to bind Cyp A. In conclusion, we showed that the decreased
38 infectivity of HIV-1-N74D in CD4⁺ T cells is due to a loss of Cyp A protection from
39 TRIM5 α_{hu} restriction activity.

40

41 **Keywords:** HIV-1; N74D; CPSF6; TRIM5 α_{hu} ; capsid; core; restriction

42

43 INTRODUCTION

44 Human cleavage and polyadenylation specificity factor subunit 6 (CPSF6) is a
45 nuclear protein that belongs to the serine/arginine-rich protein family. Expression of a
46 cytosolic fragment of CPSF6 [CPSF6(1–358)] was found to potently block human
47 immunodeficiency virus-1 (HIV-1) infection before the formation of 2-long terminal
48 repeat circles [1], and this inhibition of HIV-1 infection correlated with the ability of
49 CPSF6(1-358) to bind to the capsid and prevent uncoating [2-4]. The serial passaging
50 of HIV-1 in human cells overexpressing CPSF6(1-358) resulted in the generation of
51 escape-mutant viruses bearing the N74D capsid change(HIV-1-N74D) [1], and binding
52 studies of HIV-1 capsids with N74D mutations to CPSF6(1-358) demonstrated a lack of
53 binding as the mechanism for escape [1, 2]. Although the overexpression of full-length
54 CPSF6 remained nuclear and did not block HIV-1 infection, these experiments
55 functionally linked CPSF6 to the HIV-1 capsid. Knockdown or knockout of human
56 CPSF6 expression in different human cell lines did change HIV-1 integration site
57 selection [2, 5-8]. Several reports have also suggested that full-length CPSF6 may
58 facilitate the entry of the virus core into the nucleus [8-10]. The lack of a correlation
59 between the loss of CPSF6 binding to HIV-1 and decreased infectivity in human cell
60 lines indicates that cell-type specific differences in the pathways surrounding early
61 replication contribute to these discrepancies, suggesting the need to work in primary
62 human cell models.

63

64 This work used human primary cells to examine the role of CPSF6 in HIV-1
65 replication. Our experiments demonstrated that the HIV-1 capsid mutations N74D or

66 A77V affected the capsid's ability to interact with CPSF6, as displayed by infectivity
67 phenotypes in human primary peripheral blood mononuclear cells (PBMCs) and CD4⁺ T
68 cells. When compared with the wild-type virus, HIV-1-N74D demonstrated decreased
69 infectivity, but HIV-1-A77V infectivity was less affected.

70 These different mutant virus infectivity phenotypes suggested that the reduced
71 primary cell infectivity observed in the case of HIV-1-N74D was not likely to be due to a
72 CPSF6-binding defect. To test this hypothesis directly, we challenged CPSF6-depleted
73 human primary CD4⁺ T cells with HIV-1-N74D and HIV-1-A77V. Remarkably, the
74 reduced HIV-1-N74D infectivity in human primary cells did not change with depleted
75 CPSF6 expression, suggesting that the loss of capsid-CPSF6 interactions did not
76 account for the decreased infectivity of this mutant virus. One possibility is that a
77 different protein may be responsible for reduced HIV-1-N74D infectivity. Recently, we
78 and others have demonstrated that cyclophilin A (Cyp A) protects the HIV-1 core from
79 restriction by the human tripartite motif-containing protein 5 α (TRIM5 α_{hu}) in primary
80 CD4⁺ T cells. To test whether TRIM5 α_{hu} is involved in decreased HIV-1-N74D infectivity,
81 we challenged TRIM5 α_{hu} -depleted human primary CD4⁺ T cells with HIV-1-N74D.
82 Interestingly, we observed that TRIM5 α_{hu} depletion rescued HIV-1-N74D infectivity,
83 suggesting that TRIM5 α_{hu} is responsible for the restriction observed in human primary T
84 cells. Because Cyp A protects the core from restriction by TRIM5 α_{hu} , we also tested the
85 ability of the N74D mutation-containing capsids to bind to Cyp A. We found that these
86 capsids lost their ability to interact with Cyp A, which may explain the reason that HIV-1-
87 N74D is restricted by TRIM5 α_{hu} . Overall, our results show that the HIV-1-N74D mutant
88 virus is restricted by TRIM5 α_{hu} due to an inability to bind Cyp A.

89

90 **RESULTS**

91 **HIV-1-N74D exhibits defective infectivity of primary CD4⁺ T cells.** To test the role of
92 capsid-CPSF6 interactions during the infection process, we challenged dog and human
93 cell lines with HIV-1 viruses containing the capsid mutations N74D and A77V (both of
94 which prevent capsid interactions with CPSF6). Infectivity of wild-type and mutant HIV-
95 1_{NL4-3}Δenv, pseudotyped with vesicular stomatitis virus G (VSV-G) envelopes
96 expressing green fluorescent protein (GFP) as an infection reporter, were normalized
97 using p24 levels. HIV-1-N74D-GFP viruses showed a defect on infectivity when
98 compared to wild type viruses when infecting the lung human cell line A549 or the
99 Jurkat T cell line (Figure 1). HIV-1-A77V-GFP viruses showed a lesser defect when
100 compared to HIV-1-N74D-GFP viruses. By contrast, the infectivity defect of HIV-1-
101 N74D-GFP viruses was not observed in the canine cell Cf2Th, which do not express a
102 TRIM5 α orthologues (Figure 1).

103 Next we tested whether these infectivity defects are present in human primary
104 cells. As shown in Figure 2A, HIV-1-N74D-GFP showed a defect in PBMC infections
105 compared with wild-type HIV-1 in at least 3 donors. However, HIV-1-A77V-GFP
106 exhibited a minor infectivity defect when compared with HIV-1-N74D-GFP viruses.
107 Similar results were observed when we challenged human primary CD4⁺ T cells
108 obtained from three independent donors with HIV-1-N74D-GFP and HIV-1-A77V-GFP
109 (Figure 2B). As both N74D and A77V capsid mutants lost their ability to bind to CPSF6
110 (data not shown), the results suggested that the decreased infectivity of HIV-1-N74D-

111 GFP in primary CD4⁺ T cells is likely due to reasons other than loss of binding to
112 CPSF6.

113

114 **Depletion of CPSF6 in human primary CD4⁺ T cells does not affect HIV-1**

115 **infectivity.** To test the role of CPSF6 in HIV-1 infection of human primary cells, we
116 challenged CPSF6-depleted CD4⁺ T cells with wild-type and mutant HIV-1. As shown in
117 Figure 3A, CRISPR-Cas9 ribonucleoprotein complexes (crRNPs) containing the anti-
118 CPSF6 guide RNA (gRNA) #5 and #6 completely depleted the expression of CPSF6 in
119 human primary CD4⁺ T cells. As a control, we also knocked out the expression of
120 CXCR4. Similar to the results above, CPSF6 depletion did not affect wild-type HIV-1
121 infectivity in human primary cells (Figure 3B). In addition, depletion of CPSF6 did not
122 affect the infectivity of either HIV-1-N74D-GFP or HIV-1-A77V-GFP. These experiments
123 demonstrated that depletion of CPSF6 in human primary cells did not affect HIV-1
124 infectivity, suggesting that the reduced infectivity of HIV-1-N74D was not due to blocked
125 virus interactions with CPSF6.

126

127 **Depletion of TRIM5_{α_{hu}} in human primary CD4⁺ T cells rescues HIV-1-N74D**

128 **infectivity.** We and others have previously demonstrated that Cyp A protects the HIV-1
129 core from TRIM5_{α_{hu}} restriction in human primary CD4⁺ T cells [11, 12]. Therefore, we
130 hypothesized that TRIM5_{α_{hu}} may decrease the infectivity of HIV-1-N74D in human
131 primary cells. To test this hypothesis, we challenged TRIM5_{α_{hu}}-depleted human primary
132 CD4⁺ T cells with HIV-1-N74D-GFP. crRNPs containing the anti-TRIM5_{α_{hu}} gRNA #6
133 and #7 completely depleted the expression of endogenous TRIM5_{α_{hu}} in human primary

134 CD4⁺ T cells (Figure 4A). As shown in Figure 4B, the depletion of TRIM5 α_{hu} rescued
135 HIV-1-N74D-GFP infectivity in CD4⁺ T cells. These results suggested that TRIM5 α_{hu}
136 restricted HIV-1-N74D in human CD4⁺ T cells. Interestingly, small infectivity changes
137 were observed for HIV-1-A77V in TRIM5 α_{hu} -depleted cells, suggesting that this virus is
138 not restricted by TRIM5 α_{hu} .

139

140 **N74D-stabilized capsids bind to TRIM5 α_{hu} but do not interact with Cyp A.** If

141 TRIM5 α_{hu} restriction occurs after cells are infected by HIV-1-N74D, it implies that Cyp A
142 is no longer protecting the core. To test this hypothesis, we assessed the abilities of
143 TRIM5 α_{hu} and Cyp A to bind to N74D-stabilized capsid tubes using a capsid binding
144 assay [13]. As shown in Figure 5, TRIM5 α_{hu} bound with increased affinity to stabilized
145 N74D capsid tubes than to wild-type tubes. Interestingly, TRIM5 α_{hu} bound to A77V-
146 stabilized tubes and wild-type tubes in a similar manner. Results from these ancillary
147 experiments support the idea that the infectivity defect observed for HIV-1-N74D is due
148 to an increase in TRIM5 α_{hu} binding to N74D capsids compared with that to wild-type
149 capsids. We have previously shown that when Cyp A was not expressed in primary
150 CD4⁺ T cells, TRIM5 α_{hu} binding to capsid increased [11]; therefore, we tested the ability
151 of Cyp A to bind to N74D-stabilized capsid tubes. As shown in Figure 5, Cyp A did not
152 bind to N74D-stabilized capsid tubes, although it bound to wild-type capsid tubes.
153 These results showed that N74D capsids were not protected by Cyp A, leading to
154 TRIM5 α_{hu} binding and restriction. Interestingly, Cyp A did not bind to A77V-stabilized
155 tubes.

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158

159 **DISCUSSION**

160 Here we have shown that the infectivity defect observed for HIV-1-N74D viruses
161 in CD4⁺ T cells is due to its inability to interact with Cyp A, which exposes the viral core
162 to TRIM5 α_{hu} binding and restriction. These results concur with the idea that Cyp A plays
163 an important role in the protection of the HIV-1 core in the early stages of infection [11,
164 12], and indicate once again that Cyp A may be essential in the early stages of HIV-1
165 infection to ensure protection of the core from restriction factors or cellular conditions
166 that may affect infection. These results are in contrast to the current notion that the
167 infectivity defect of N74D is due to its loss of CPSF6 binding. Although capsids bearing
168 the N74D change do not interact with CPSF6, the infectivity defect that HIV-1-N74D
169 viruses exhibit is due to a decrease in Cyp A binding with a concomitant gain of
170 TRIM5 α_{hu} binding, which restricts infection.

171 While an intact HIV-1 core has more than ~1200 binding sites for Cyp A, the
172 actual number of sites occupied by Cyp A during an infection is not known; however, it
173 is reasonable to think that binding of one or two Cyp A molecules per hexamer would be
174 sufficient to prevent the binding of restriction factors such as TRIM5 α_{hu} by steric
175 hindrance. So theoretically, only two Cyp A molecules per hexamer would be needed to
176 ensure that infection is productive. Interestingly, residue N74 in the capsid structure is
177 distantly located from the Cyp A binding loop, suggesting that an overall structural shift
178 may be occurring in order to prevent Cyp A binding. An alternative explanation is that
179 the N74D mutation may affect core breathing, and consequently, inhibit the binding of

180 Cyp A to the core [14]. One of the implications of this study is that interactions between
181 Cyp A and capsid mutants should be considered when trying to understand HIV-1
182 infectivity defects involving human primary cells.

183 The infectivity defect of HIV-1-A77V viruses was not very pronounced when
184 compared to HIV-1-N74D viruses. In addition, depletion of TRIM5 α_{hu} did not rescue the
185 infectivity defect of HIV-1-A77V. In agreement, A77V stabilized capsid tubes did not
186 showed an increase in binding to TRIM5 α_{hu} , but lost binding to Cyp A. One possibility is
187 that HIV-1-A77V viruses are defective for a different reason, which agrees with the
188 experiments showing that HIV-1-A77V viruses can replicate in CD4⁺ T cells when
189 compared to HIV-1-N74D [15].

190 While this work was ongoing, TRIM34 has also been shown to be important for
191 decreased HIV-1-N74D infectivity in primary CD4⁺ T cells [16]. Taken together, these
192 results suggest that TRIM5 α_{hu} may be working together with TRIM34 to reduce HIV-1-
193 N74D infectivity. We demonstrated previously that TRIM5 α proteins can form higher-
194 order, self-associating complexes which are essential for TRIM5 α -based restriction of
195 retroviruses [17, 18]. In addition, these studies showed that TRIM5 α proteins can also
196 form higher-order complexes with TRIM orthologs such as TRIM34 and TRIM6 [19]. It is
197 possible that TRIM5 α_{hu} forms higher-order complexes with TRIM34 in order to bind and
198 restrict HIV-1-N74D viruses, as we have previously suggested [20].

199 These results highlight the importance of HIV-1 core-Cyp A interactions during
200 productive HIV-1 infection and indicate that Cyp A is an essential cofactor for HIV-1
201 replication in human primary CD4⁺ T cells.

202

203

204 **MATERIALS AND METHODS**

205 **Infection using HIV-1-GFP reporter viruses**

206 Recombinant HIV-1 strains (e.g., HIV-1-N74D and HIV-1-A77V) expressing GFP,
207 and pseudotyped with VSV-G, were prepared as previously described [21]. All viruses
208 were titered according to p24 levels and infectivity. Viral challenges were performed in
209 24-well plates by infecting 50,000 cells (PBMCs or CD4⁺ T cells) per well. Infectivity was
210 determined by measuring the percentage of GFP-positive cells using flow cytometry
211 (BD FACSCelesta, San Jose, CA, USA).

212

213 **Capsid expression and purification**

214 The pET-11a vector was used to express HIV-1 capsid proteins containing the
215 A14C and E45C mutations. Point mutations N74D and A77V were introduced using the
216 QuikChange II site-directed mutagenesis kit (Stratagene) according to the
217 manufacturer's instructions. All proteins were expressed in *Escherichia coli* one-shot
218 BL21star (DE3) cells (Invitrogen, Carlsbad, CA, USA), as previously described [13].
219 Briefly, cells were inoculated in Luria-Bertani medium and cultured at 30°C until mid-log
220 phase (Absorbance at 600 nm, 0.6–0.8). Protein expression was induced with 1 mM
221 isopropyl-β-d-thiogalactopyranoside overnight at 18°C. Cells were harvested by
222 centrifugation at 5,000 × g for 10 min at 4°C, and pellets were stored at –80°C until
223 purification. Purification of capsids was carried out as follows. Pellets from two-liter
224 cultures were lysed by sonication (Qsonica microtip: 4420; A = 45; 2 min; 2 sec on; 2
225 sec off for 12 cycles), in 40 ml of lysis buffer (50 mM Tris pH = 8, 50 mM NaCl, 100 mM

226 β -mercaptoethanol, and Complete ethylenediaminetetraacetic acid (EDTA)-free
227 protease inhibitor tablets). Cell debris was removed by centrifugation at 40,000 \times g for
228 20 min at 4°C. Proteins from the supernatant were precipitated by incubation with one-
229 third the volume of saturated ammonium sulfate containing 100 mM β -mercaptoethanol
230 for 20 min at 4°C, and centrifugation at 8,000 \times g for 20 min at 4°C. Precipitated
231 proteins were resuspended in 30 ml of buffer A (25 mM 2-(N-morpholino) ethanesulfonic
232 acid (MES), pH 6.5, and 100 mM β -mercaptoethanol) and sonicated 2–3 times (Qsonica
233 microtip: 4420; A = 45; 2 min; 1 sec on; 2 sec off). The protein sample was dialyzed
234 three times in buffer A (2 h, overnight, and 2 h), sonicated, diluted in 500 ml of buffer A,
235 and then separated sequentially on a 5-ml HiTrap Q HP column followed by a 5-ml
236 HiTrap SP FF column (GE Healthcare), which were both pre-equilibrated with buffer A.
237 Capsid proteins were eluted from the HiTrap SP FF column using a linear gradient of
238 concentrations ranging from 0–2 M NaCl. The eluted fractions that had the highest
239 protein levels were selected based on absorbance at 280 nm. Pooled fractions were
240 dialyzed three times (2 h, overnight, and 2 h) in storage buffer (25 mM MES, 2 M NaCl,
241 20 mM β -mercaptoethanol). Samples were concentrated to 20 mg/ml using Centriprep
242 Centrifugal Filter Units and stored at –80°C.

243

244 **Assembly of stabilized HIV-1 capsid tubes**

245 One milliliter of monomeric capsid (5 mg/ml) was dialyzed in SnakeSkin dialysis
246 tubing (10K MWCO, Thermo Scientific, Waltham, MA, USA) using a buffer that was high
247 in salt and contained a reducing agent (Buffer 1: 50 mM Tris, pH 8, 1 M NaCl, 100 mM
248 β -mercaptoethanol) at 4°C for 8 h. The protein was then dialyzed using the same buffer

249 without the reducing agent β -mercaptoethanol (Buffer 2: 50 mM Tris, pH 8, 1 M NaCl) at
250 4°C for 8 h. The absence of β -mercaptoethanol in the second dialysis allowed the
251 formation of disulfide bonds between Cysteine 14 and the 45 inter-capsid monomers in
252 the hexamer. Finally, the protein was dialyzed using Buffer 3 (20 mM Tris, pH 8, 40 mM
253 NaCl) at 4°C for 8 h. Assembled complexes were kept at 4°C for up to 1 month.

254

255 **Capsid binding assay protocol**

256 Human HEK293T cells were transfected for 24 h with a plasmid expressing the
257 protein of interest (TRIM5 α_{hu}). The culture media was completely removed and cells
258 were scraped off the plate and lysed in 300 μ l of capsid binding buffer (CBB: 10 mM
259 Tris, pH 8, 1.5 mM MgCl₂, 10 mM KCl). Cells were rotated for 15 min at 4°C and then
260 centrifuged to remove cellular debris (21,000 \times g, 15 min, 4°C). Cell lysates were
261 incubated with stabilized HIV-1 capsid tubes for 1 h at 25°C. The stabilized HIV-1
262 capsid tubes were then centrifuged at 21,000 \times g for 2 min. Pellets were washed 2–3
263 times by resuspension and centrifugation in CBB or phosphate-buffered saline (PBS).
264 Pellets were resuspended in 1 \times Laemmli buffer and analyzed by western blotting using
265 an anti-p24 antibody and other appropriate antibodies.

266

267 **Preparation of PBMCs and CD4⁺ T cells**

268 PBMCs from healthy-donor whole blood were isolated by density gradient
269 centrifugation using Ficoll-Paque Plus (GE Health Care, Chicago, IL, USA). Whole
270 blood (40 ml) was centrifuged at 300 \times g for 10 min, and the plasma layer was removed
271 and replaced with Hank's Balanced Salt solution (HBSS; Sigma Aldrich, St. Louis, MO,

272 USA). The blood sample was then diluted 1:2 with HBSS, and 20 ml of the diluted
273 sample was layered on top of 20 ml Ficoll-Paque Plus and centrifuged at $300 \times g$ for 30
274 min. The resulting buffy coat layer was collected, washed twice with HBSS, and
275 resuspended in Roswell Park Memorial Institute (RPMI) medium containing 10%
276 (vol/vol) fetal bovine serum (FBS) and 1% (vol/vol) penicillin-streptomycin, and activated
277 with IL-2 (100 U/ml) (Human IL-2; Cell Signaling Technology, #8907SF) and
278 phytohemagglutinin (1 $\mu\text{g/ml}$) for 3 days. CD4^+ T cells were obtained via negative
279 selection from PBMCs using a human CD4^+ T-cell isolation kit (MACS Miltenyi Biotec,
280 #130-096-533, Bergisch Gladbach, Germany). PBMCs (1×10^7 total cells) were
281 resuspended in 40 μl of CD4^+ T-cell isolation buffer (PBS, pH 7.2, 0.5% bovine serum
282 albumin (BSA), and 2 mM EDTA). CD4^+ T-cell biotin-antibody cocktail (10 μl) was then
283 added to the PBMCs and incubated at 4°C for 5 min. CD4^+ T-cell isolation buffer (30 μl)
284 and CD4^+ T-cell microbead cocktail (20 μl) were then added and further incubated for 10
285 min at 4°C . Depending on the number of PBMCs isolated, either an LS or MS column
286 attached to a Magnetic Activated Cell Sorting Separator was prewashed using 3 ml or 6
287 ml of ice-cold CD4^+ T-cell isolation buffer, respectively. The PBMC suspension was
288 added to the column and the flow-through was collected in a 15-ml tube. The LS or MS
289 column was then washed (3 ml or 6 ml, respectively, with ice-cold CD4^+ T-cell isolation
290 buffer), and the flow-through was collected. The newly isolated CD4^+ T cells were then
291 centrifuged at $800 \times g$ for 5 min and resuspended in RPMI medium supplemented with
292 IL-2 (100 U/ml).

293

294 **CRISPR-Cas9 knockouts in primary CD4^+ T cells**

295 Detailed protocols for the production of CRISPR-Cas9 ribonucleoprotein
296 complexes (crRNPs) and primary CD4⁺ T-cell editing have been previously published
297 [22, 23]. Briefly, lyophilized CRISPR RNA (crRNA) and trans-activating crRNA
298 (tracrRNA; Dharmacon, Lafayette, CO, USA) were each resuspended at a
299 concentration of 160 μM in 10 mM Tris-HCl (pH 7.4), and 150 mM KCl. Five microliters
300 of 160 μM crRNA was then mixed with 5 μl of 160 μM tracrRNA and incubated for 30
301 min at 37°C. The gRNA:tracrRNA complexes were then mixed gently with 10 μl of 40
302 μM purified Cas9-NLS protein (UC-Berkeley Macrolab) to form crRNPs. Complexes
303 were aliquoted and frozen in 0.2-ml PCR tubes (USA Scientific, Ocala, FL, USA) at –
304 80°C until further use. crRNA guide sequences used in this study were a combination of
305 sequences derived from the Dharmacon predesigned Edit-R library for gene knockouts,
306 and custom-ordered sequences as indicated.

307 PBMCs were isolated by density gradient centrifugation using Ficoll-Paque Plus
308 (GE Health Care, #17-1440-02). PBMCs were washed thrice with 1× PBS to remove
309 platelets and resuspended at a final concentration of 5 × 10⁸ cells/ml in 1× PBS, 0.5%
310 BSA, and 2 mM EDTA. Bulk CD4⁺ T cells were subsequently isolated from PBMCs by
311 magnetic negative selection using an EasySep Human CD4⁺ T Cell Isolation Kit
312 (STEMCELL, per manufacturer's instructions). Isolated CD4⁺ T cells were suspended in
313 complete RPMI medium, consisting of RPMI-1640 (Sigma Aldrich) supplemented with 5
314 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Corning, Corning, NY, USA),
315 50 μg/ml penicillin/streptomycin (Corning), 5 mM sodium pyruvate (Corning), and 10%
316 FBS (Gibco). Media was supplemented with 20 IU/ml IL-2 (Miltenyi) immediately before
317 use. For activation, bulk CD4⁺ T cells were immediately plated on anti-CD3-coated

318 plates [coated for 12 hours at 4°C with 20 µg/ml anti-CD3 antibody (UCHT1, Tonbo
319 Biosciences)] in the presence of 5 µg/ml soluble anti-CD28 antibody (CD28.2, Tonbo
320 Biosciences). Cells were stimulated for 72 h at 37°C in a 5%-CO₂ atmosphere prior to
321 electroporation. After stimulation, cell purity and activation were verified by CD4/CD25
322 immunostaining and flow cytometry as previously described [22].

323 After three days of stimulation, cells were resuspended and counted. Each
324 electroporation reaction consisted of between 5×10^5 and 1×10^6 T cells, 3.5 µl RNPs,
325 and 20 µl of electroporation buffer. crRNPs were thawed to room temperature.
326 Immediately prior to electroporation, cells were centrifuged at 400 × g for 5 minutes, the
327 supernatant was removed by aspiration, and the pellet was resuspended in 20 µl of
328 room temperature P3 electroporation buffer (Lonza, Basel, Switzerland) per reaction.
329 Cell suspensions (20 µl) were then gently mixed with each RNP and aliquoted into a 96-
330 well electroporation cuvette for nucleofection with the 4D 96-well shuttle unit (Lonza)
331 using pulse code EH-115. Immediately after electroporation, 100 µl of prewarmed media
332 without IL-2 was added to each well and cells were allowed to rest for 30 min in a cell-
333 culture incubator at 37°C. Cells were subsequently moved to 96-well flat-bottomed
334 culture plates prefilled with 100 µl warm complete media with IL-2 at 40 U/ml (for a final
335 concentration of 20 U/ml) and anti-CD3/anti-CD2/anti-CD28 beads (T cell Activation and
336 Stimulation Kit, Miltenyi) at a 1:1 bead:cell ratio. Cells were cultured at 37°C in a 5%-
337 CO₂ atmosphere, dark, humidified cell-culture incubator for four days to allow for gene
338 knockout and protein clearance, with additional media added on day 2. To check
339 knockout efficiency, 50 µl of mixed culture was transferred to a centrifuge tube. Cells
340 were pelleted, the supernatant removed, and the pellets were resuspended in 100 µl

341 2.5× Laemmli Sample Buffer. Protein lysates were heated to 98°C for 20 min before
342 storage at –20°C until assessment by western blotting.

343 RNA guides:

Synthetic RNA / Gene Target	Guide #	Sequence	Catalog Number (Dharmacon)
Edit-R Synthetic tracrRNA	n/a	n/a	U-002005-50
Edit-R crRNA Non-targeting Control #3	3	n/a	U-007503-20
CXCR4 crRNA	1	GAAGCGTGATGACAAAGAGG	Custom sequence
CPSF6 crRNA	5	GGACCACATAGACATTTACG	CM-012334-05
CPSF6 crRNA	6	ATATATTGGAAATCTAACAT	Custom sequence
TRIM5alpha crRNA	6	AAGAAGTCCATGCTAGACAA	Custom sequence
TRIM5alpha crRNA	7	GTTGATCATTGTGCACGCCA	Custom sequence

344

345

346 **QUANTIFICATION AND STATISTICAL ANALYSES**

347 Statistical analyses were performed using unpaired t-tests. Sample numbers,
348 number of replicates, and *p* values are indicated in corresponding figure legends.

349 Quantification of western blot band intensities was performed using ImageJ. For all
350 experiments, means and standard deviations were calculated using GraphPad Prism
351 7.0c.

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353

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357

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456
457

458 **FIGURE LEGENDS**

459

460 **Figure 1. HIV-1-N74D exhibits an infectivity defect in human cell lines but not in**
461 **dog cell lines.** Human lung A549 cells, human Jurkat T cells, or dog thymus Cf2Th
462 cells were challenged with increasing amounts of the indicated p24-normalized WT and
463 mutant HIV-1 viruses. Infectivity was determined at 48 h post-challenge by measuring
464 the percentage of GFP-positive cells. Experiments were repeated three times and a
465 representative experiment is shown.

466

467 **Figure 2. HIV-1-N74D exhibits an infectivity defect in primary PBMCs and CD4⁺ T**
468 **cells.** Human primary PBMCs **(A)** or purified CD4⁺ T cells **(B)** from healthy donors were
469 challenged with increasing amounts of p24-normalized HIV-1-GFP, HIV-1-N74D-GFP,
470 or HIV-1-A77V-GFP. Infectivity was determined at 72 h post-challenge by measuring
471 the percentage of GFP-positive cells. Experiments were repeated three times per donor,
472 and a representative experimental result is shown. Statistical analysis was performed
473 using an intermediate value taken from the infection curves (right panel). ** indicates P-
474 value < 0.001, *** indicates P-value < 0.0005, **** indicates P-value < 0.0001 as
475 determined by using the unpaired t-test.

476

477

478 **Figure 3. Depleted CPSF6 expression in human primary CD4⁺ T cells does not**
479 **affect HIV-1 infectivity. (A)** Human primary CD4⁺ T cells from two different donors had
480 CPSF6 expression knocked out using the CRISPR/Cas9 system, as described in

481 Methods. Briefly, CD4⁺ T cells were electroporated using two different guide RNAs
482 (gRNAs) against CPSF6 (gRNA #5 and #6) together with the Cas9 protein. At 72 h
483 post-electroporation, endogenous expression of CPSF6 in CD4⁺ T cells was analyzed
484 by western blotting using an antibody against CPSF6. For controls, a gRNA against
485 CXCR4 and a non-targeting gRNA were electroporated. Expression of GAPDH was
486 used as a loading control. Similar results were obtained using two different donors, and
487 a representative blot is shown. **(B)** Human primary CD4⁺ T cells depleted for CPSF6
488 expression were challenged with increasing amounts of p24-normalized HIV-1-GFP,
489 HIV-1-N74D-GFP, or HIV-1-A77V-GFP. Infectivity was determined at 72 h post-
490 challenge by measuring the percentage of GFP-positive cells. Experiments were
491 repeated three times per donor, and a representative experimental result is shown.
492 Statistical analysis was performed using an intermediate value taken from the infection
493 curves (bottom panels). ** indicates P-value < 0.001, *** indicates P-value < 0.0005 as
494 determined by using the unpaired t-test.

495

496

497 **Figure 4. TRIM5 α_{hu} depletion in human primary CD4⁺ T cells rescues HIV-1-N74D**
498 **infectivity. (A)** Human primary CD4⁺ T cells from three different donors had TRIM5 α_{hu}
499 expression knocked out using the CRISPR/Cas9 system, as described in Methods.
500 Briefly, CD4⁺ T cells were electroporated using two different guide RNAs (gRNAs)
501 against TRIM5 α_{hu} (gRNA #6 and #7) together with the Cas9 protein. At 72 h post-
502 electroporation, the endogenous expression of TRIM5 α_{hu} in CD4⁺ T cells was analyzed
503 by western blotting using an antibody against TRIM5 α_{hu} . For controls, a gRNA against

504 CXCR4, and a non-targeting gRNA were electroporated. Expression of GAPDH was
505 used as a loading control. Similar results were obtained using two different donors, and
506 a representative blot is shown. **(B)** Human primary CD4⁺ T cells depleted for TRIM5 α_{hu}
507 expression were challenged with increasing amounts of p24-normalized HIV-1-GFP,
508 HIV-1-N74D-GFP, or HIV-1-A77V-GFP. Infectivity was determined at 72 h post-
509 challenge by measuring the percentage of GFP-positive cells. Experiments were
510 repeated three times per donor, and a representative experimental result is shown.
511 Statistical analysis was performed using an intermediate value taken from the infection
512 curves (bottom panels). * indicates P-value < 0.005, ** indicates P-value < 0.001, ***
513 indicates P-value < 0.0005, NS indicates not significant as determined by using the
514 unpaired t-test.

515

516

517

518 **Figure 5. N74D-stabilized capsids bind to TRIM5 α_{hu} but do not interact with Cyp**

519 **A.** Human 293T cells were transfected with plasmids expressing TRIM5 α_{hu} -
520 hemagglutinin (HA). Post-transfection (24 h), cells (INPUT) were lysed in capsid binding
521 buffer (CBB) as described in Methods. Cell extracts containing TRIM5 α_{hu} -HA were then
522 mixed with 10 μ l of either stabilized wild-type, N74D, or A77V capsid tubes (5 mg/ml).
523 Mixtures were incubated for 1 h at room temperature. Stabilized HIV-1 capsid tubes
524 were collected by centrifugation and washed twice using CBB. Pellets were
525 resuspended in 1 \times Laemmli buffer (BOUND). INPUT and BOUND fractions were then
526 analyzed by western blotting using anti-HA, anti-Cyp A, and anti-p24 antibodies.

527 Experiments were repeated three times, and a representative experimental result is
528 shown. The BOUND fraction relative to the INPUT fraction for three independent
529 experiments (with standard deviation) is shown. * indicates a p-value < 0.005, ****
530 indicates a p-value < 0.0001, and NS indicates no significant difference as determined
531 by unpaired t-tests.
532

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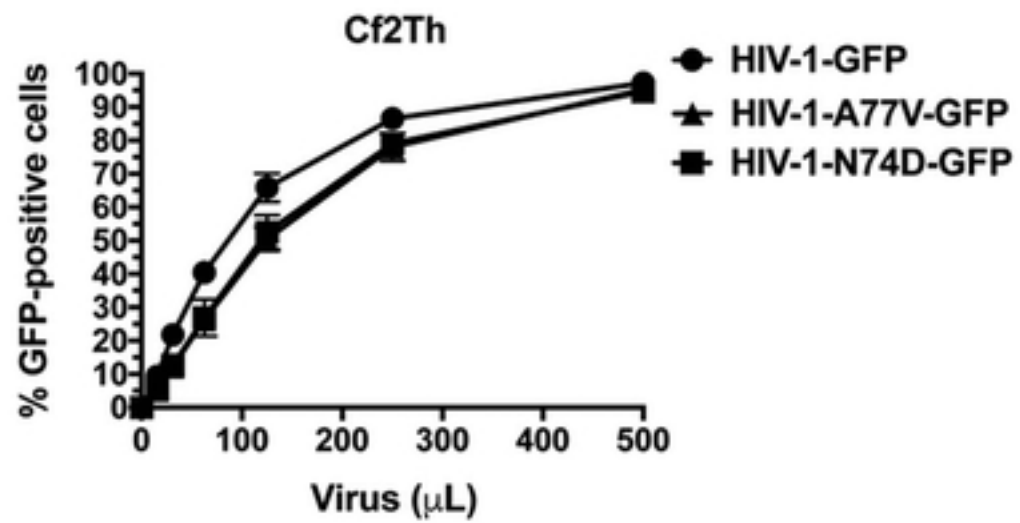
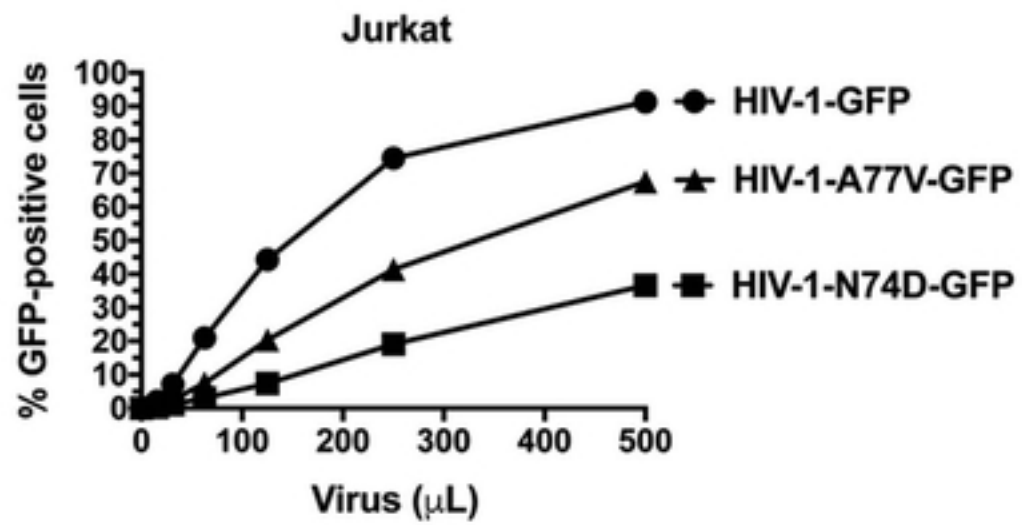
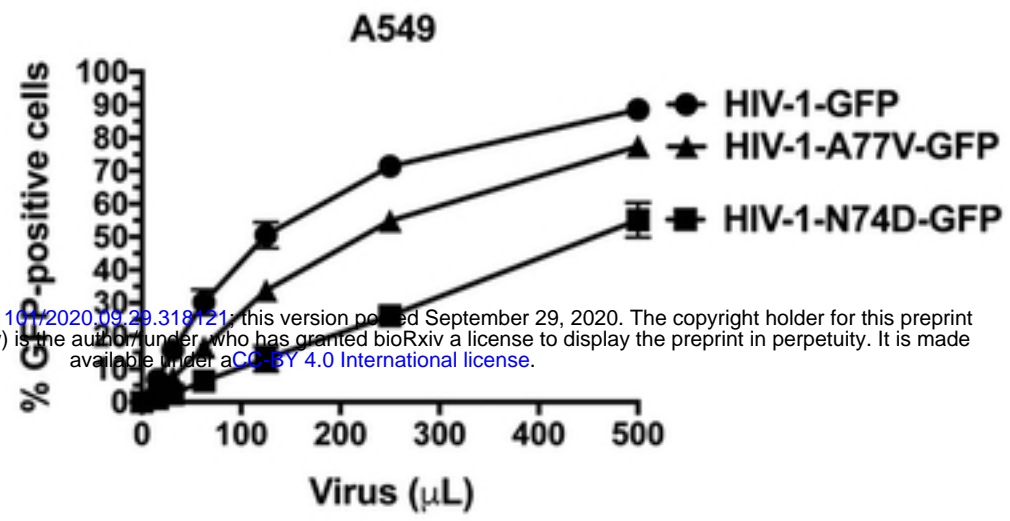
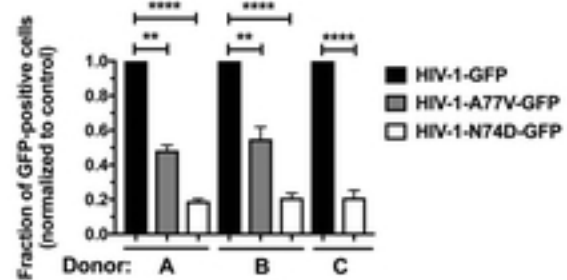
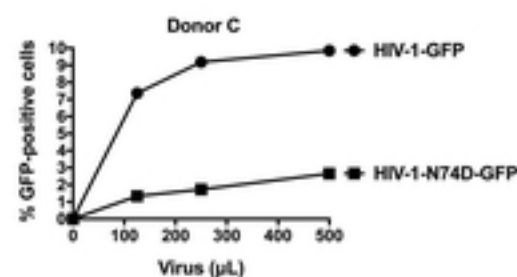
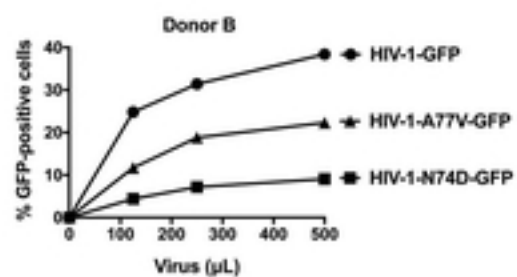
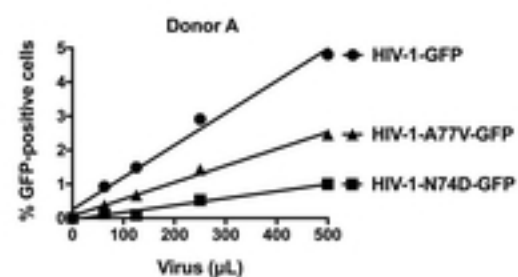


Figure 1

A PBMCs



B CD4⁺ T cells

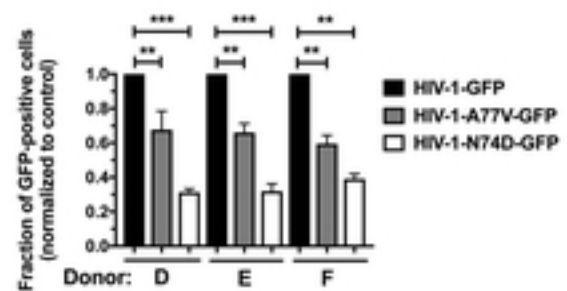
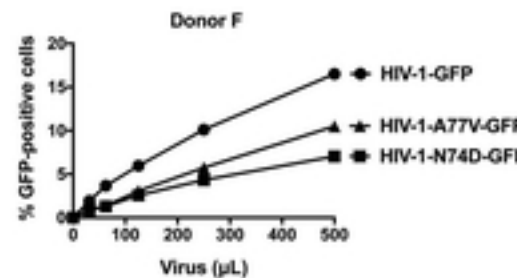
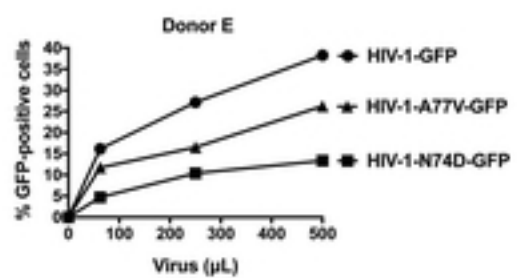
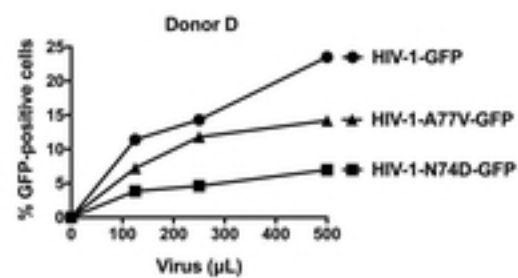


Figure 2

Figure 2

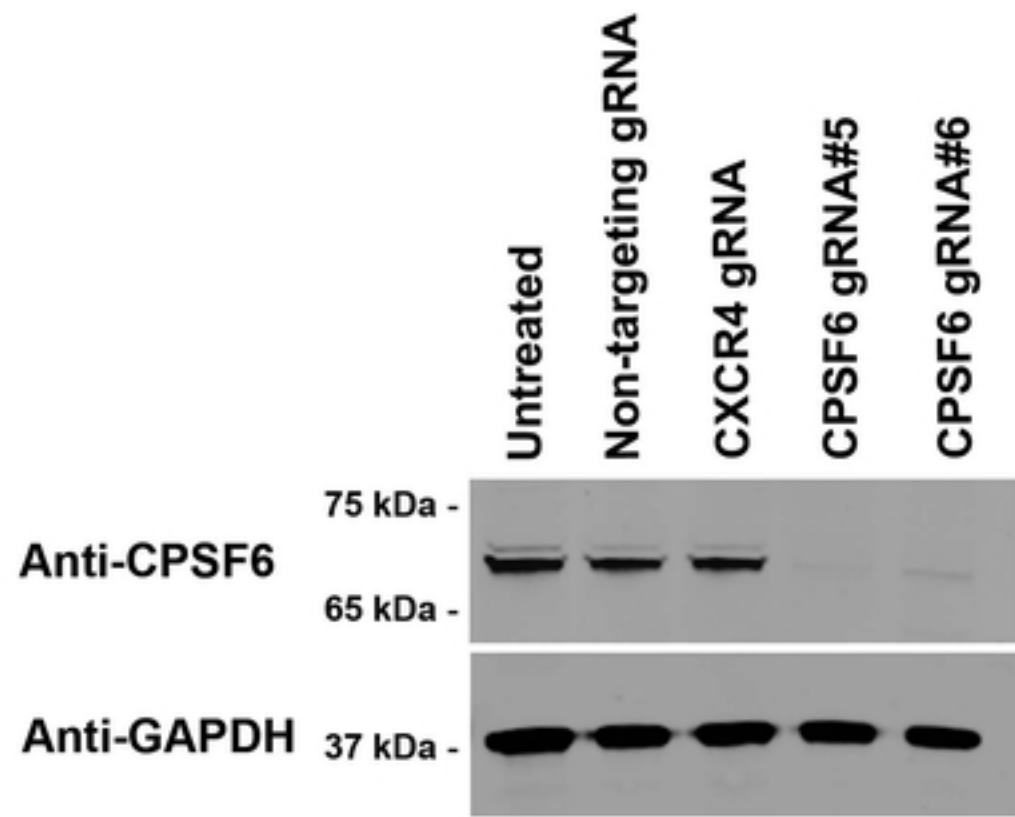
A**Figure 3**

Figure 3A

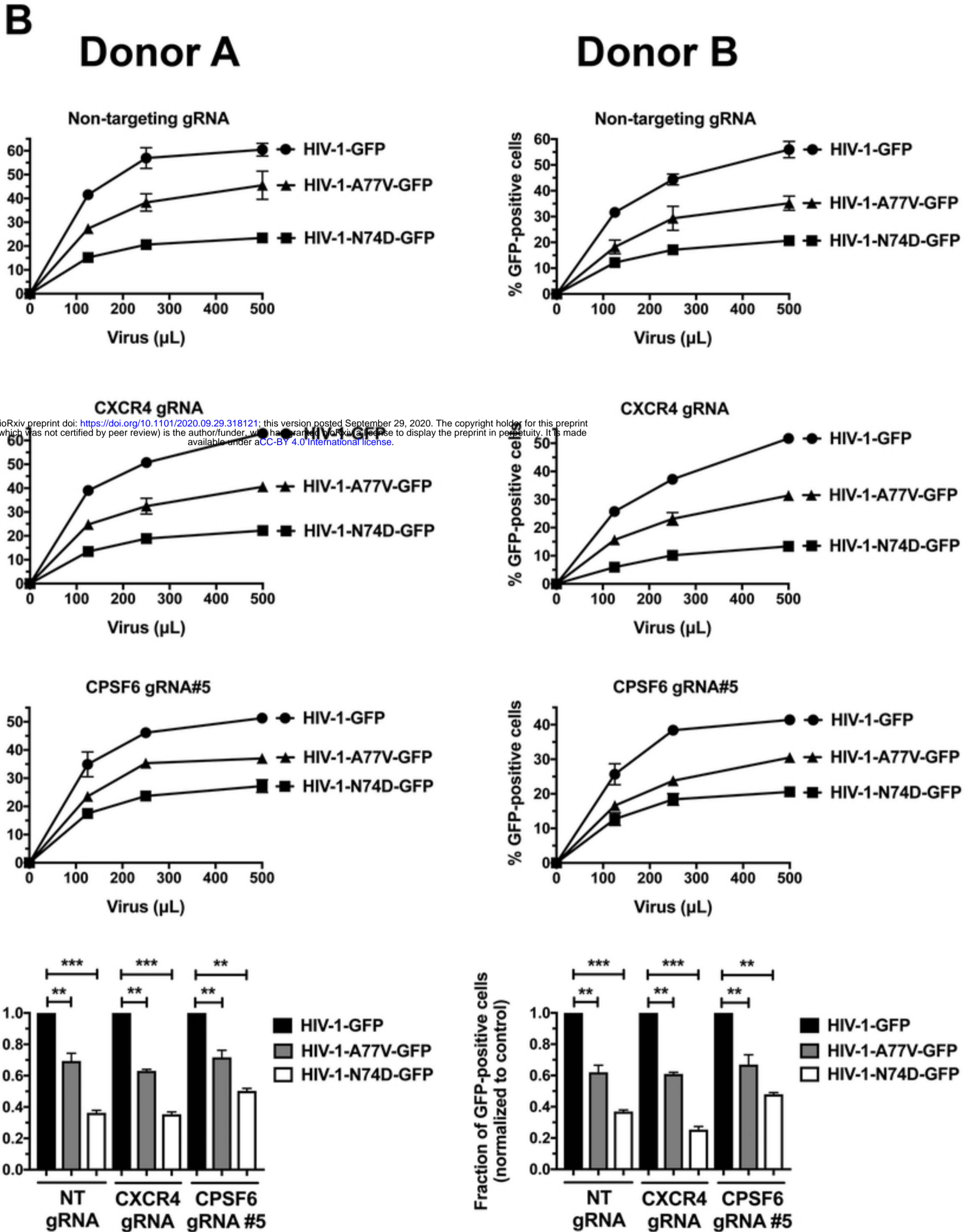
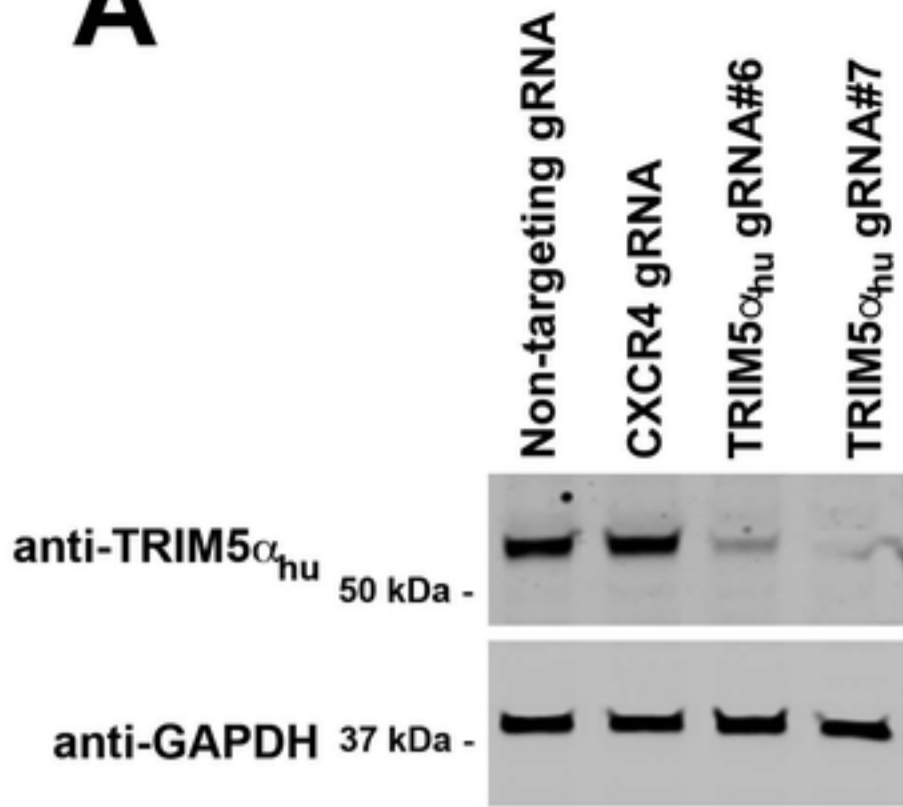
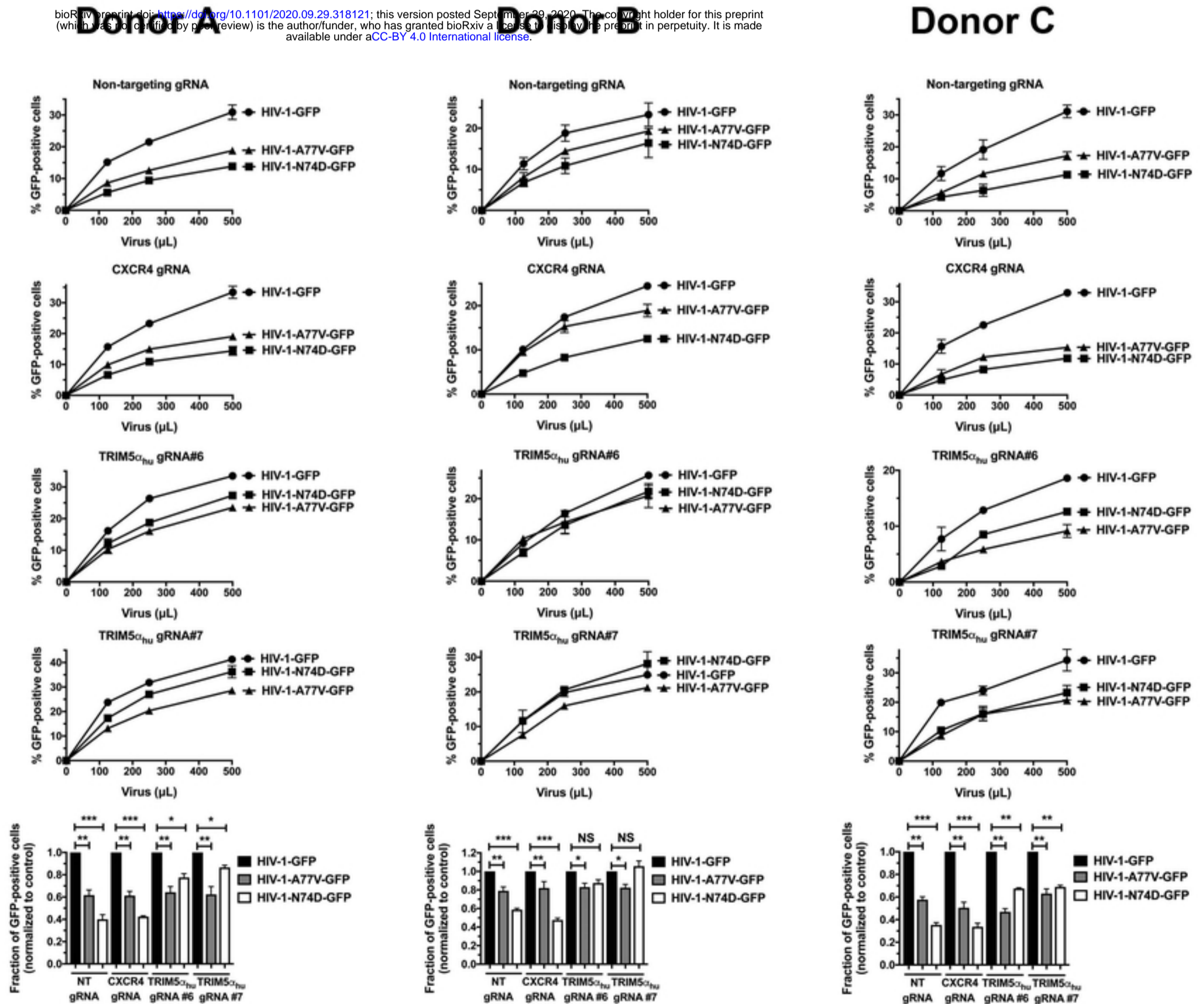


Figure 3B

A**B**

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Figure 4**Figure 4**

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HIV-1 capsid A14C/E45C: WT N74D A77V -
TRIM5 α_{hu} -HA: + + + +

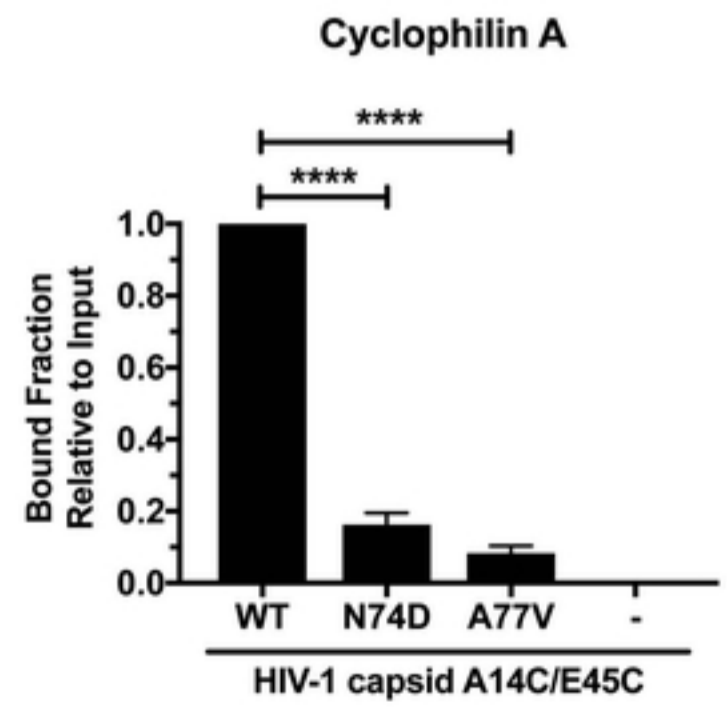
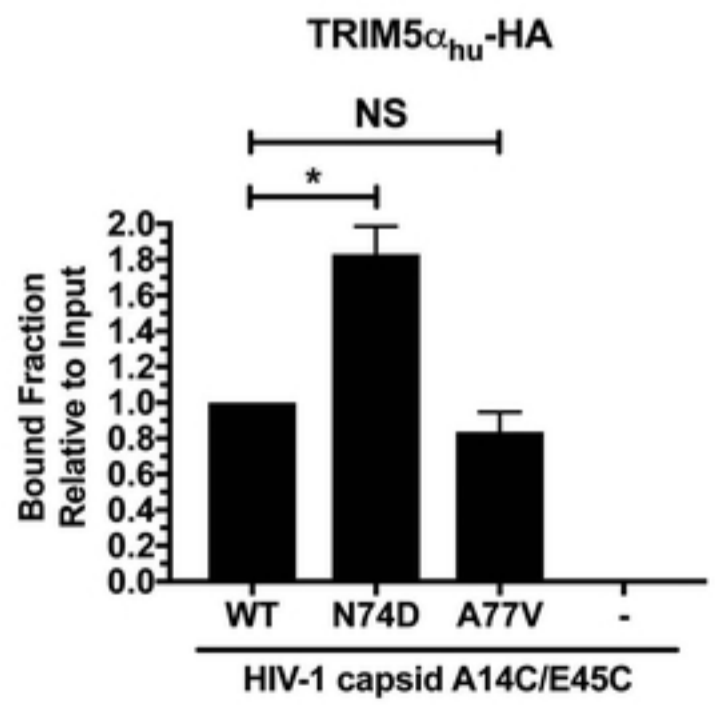
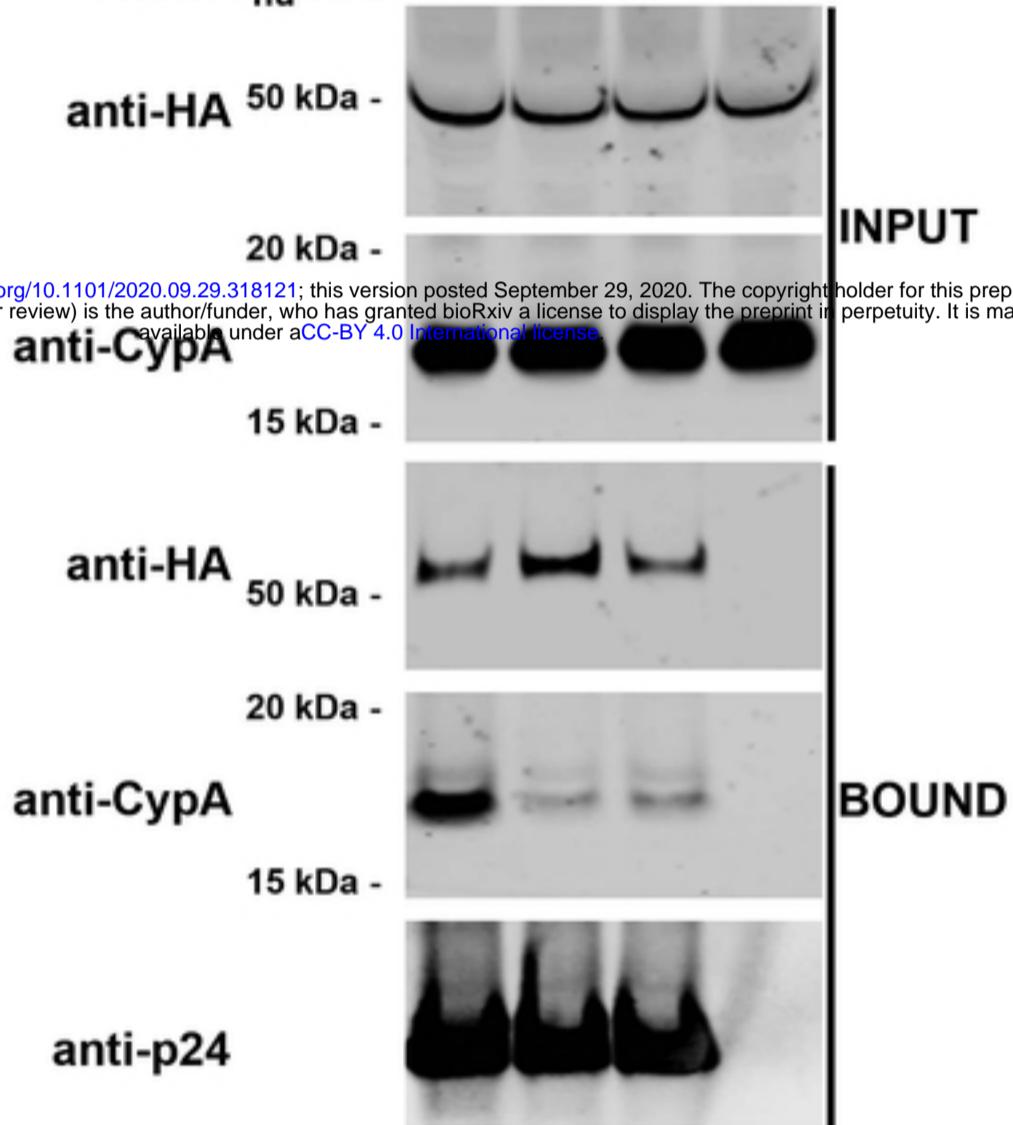


Figure 5

Figure 5