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Item Type	Preprint
Authors	Xue, Guangai;Yu, Hyun Jae;University of Massachusetts Medical School;Gres, Anna T.;Guney, Mehmet Hakan;Sarafianos, Stefan G.;Luban, Jeremy;KewalRamani, Vineet N.
Citation	<p>bioRxiv 2021.12.02.470925; doi: https://doi.org/10.1101/2021.12.02.470925 . Link to preprint on bioRxiv.</p>
DOI	10.1101/2021.12.02.470925
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Link to Item	https://hdl.handle.net/20.500.14038/30741

1 **The HIV-1 capsid core is an opportunistic nuclear import receptor**

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3 Guangai Xue^{1,6}, Hyun Jae Yu¹, Shih Lin Goh², Anna T. Gres³, Mehmet Hakan Guney², Stefan G.

4 Sarafianos^{4,5}, Jeremy Luban², and Vineet N. KewalRamani^{1,*}

5

6 ¹Basic Research Laboratory, National Cancer Institute, Frederick, MD, 21702, USA

7 ²Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA,

8 01605, USA

9 ³Bond Life Sciences Center, Chemistry, University of Missouri, Columbia, MO, 65201, USA

10 ⁴Bond Life Sciences Center, MMI, Biochemistry, University of Missouri, Columbia, MO, 65201,

11 USA

12 ⁵Laboratory of Biochemical Pharmacology, Department of Pediatrics, Emory University School of

13 Medicine, Atlanta, GA, 30322, USA

14 ⁶Present address: Department of Molecular Physiology and Biological Physics, University of

15 Virginia, Charlottesville, VA 22908, USA

16

17

18 *Corresponding author

19 email: vineet@mail.nih.gov

20 phone: 301-846-1249

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22 **The movement of viruses and other large macromolecular cargo through nuclear pore**
23 **complexes (NPCs) is poorly understood. The human immunodeficiency virus type 1 (HIV-1)**
24 **provides an attractive model to interrogate this process due to the genetic and cell biological**
25 **assays to score virus nuclear entry in living cells. Although initial studies of HIV-1 infection of**
26 **nondividing cells focused on karyophilic virion proteins, subsequent work revealed the viral**
27 **capsid (CA), the chief structural component of the pre-integration complex (PIC), to be a critical**
28 **determinant in nuclear transport¹. In support of this model, HIV-1 interactions with NPCs can**
29 **be altered through CA mutation², which makes direct contact with nucleoporins (Nups)³⁻⁵. Here**
30 **we identify Nup35, Nup153, and POM121 to coordinately support HIV-1 nuclear entry. For**
31 **Nup35 and POM121, this dependence was strongly dependent cyclophilin A (CypA) interaction**
32 **with CA. Mutation of CA or removal of soluble host factors changed the interaction with the**
33 **NPC. Collectively, these findings implicate the HIV-1 CA hexameric lattice that encapsulates the**
34 **viral genome as a macromolecular nuclear transport receptor (NTR) that exploits soluble host**
35 **factors to modulate NPC requirements during nuclear invasion.**

36 HIV-1 exhibits significant flexibility in its utilization of the NPC to access the nucleus during
37 early replication steps. Comprised of over 30 distinct proteins, the NPC incorporates six subcomplexes
38 based on stoichiometry and distribution within the NPC (Extended Data Fig. 1a) ⁶. Approximately one-
39 third of Nups contain FG-dipeptides, motifs which are targets for NTR interaction for directional
40 movement of cargo from the cytoplasm to the nucleus ⁷. Genetic and biochemical studies have
41 implicated the FG-Nups Nup153 and Nup358 as contributing to HIV-1 nuclear entry ^{5,8-11}. These Nups
42 decorate opposite poles of the NPC with tentacles of Nup358 extending into the cytoplasm from the
43 outer nuclear membrane and Nup153 projecting a basket-like structure from the inner nuclear
44 membrane into the nucleoplasm. HIV-1 with mutations in CA that prevent interaction with soluble

45 factors such as CPSF6 and CypA, exhibit a differential dependence on Nups during infection, including
46 loss of reliance on both Nup153 and Nup358. CypA interacts with CA via the P90 residue exposed on
47 loop in the N-terminal domain (NTD), and CPSF6 interacts via the N74 pocket within an NTD and C-
48 terminal domain (CTD) interface present in hexameric CA in the virion core (Extended Data Fig. 1b
49 and 1c). P90A and N74D mutations, respectively, impair CypA and CPSF6 interactions with CA. We
50 considered the possibility that CPSF6 and CypA binding to HIV-1 in the cell cytoplasm affected
51 subsequent interactions at the NPC and thus routes of nuclear entry.

52 To better understand the applicability of this interface exposure model, we performed a genetic
53 screen with small-interfering RNAs (siRNAs) targeting 32 human Nups in HeLa cells to identify those
54 that exhibited CA-dependence during infection with VSV-G pseudotyped HIV-1 vectors encoding red
55 fluorescent protein (RFP). As a positive control to inhibit infection of HIV-1 with wild-type (WT) CA,
56 TNPO3 was also depleted using siRNA⁸. Parallel infections were performed with HIV-1 CA mutant
57 (N74D and P90A) virus vectors and Moloney murine leukemia virus (MLV) vectors. Nup35, Nup153,
58 Nup358, and POM121 depletion were observed to inhibit WT HIV-1 infection but affected N74D and
59 P90A HIV-1 infection to a lesser degree (Fig. 1a). Nup35 and POM121 have not previously been
60 studied as potential HIV-1 cofactors, and notably, their depletion had little effect on MLV infection
61 (Extended Data Fig. 2). As has been previously reported^{2,11}, Nup160 depletion affected WT and CA
62 mutant HIV-1 infection, which is likely due to the critical role it plays in NPC formation.

63 By contrast, depletion of Nup62 or Nup214 elevated WT HIV-1 infection, and Nup54 and
64 Nup58 knockdown enhanced infection of both WT and CA mutant HIV-1. MLV infection was not
65 increased in Nup54, Nup58, Nup62, or Nup214 knockdown cells (Extended Data Fig. 2). Nup54,
66 Nup58, Nup62, and Nup214 are notable due to their formation of an “FG-hydrogel” that provides a
67 selective barrier to transport between cytoplasm and nucleus.

68 Because Nup35 and POM121 depletion affected WT HIV-1 infection but not CA mutant HIV-
69 1 in a pattern similar to Nup153 and Nup358, we focused efforts on these two Nups. Initial studies were
70 performed with Nup35 as it was less essential to N74D and P90A HIV-1 relative to POM121. Nup153
71 was selected over Nup358 as a control in subsequent experiments given significant cellular toxicity
72 observed after Nup358 knockdowns. Nup153 was also of interest because HIV-1 CA engages it via an
73 FG-motif.

74 To determine whether the effect of Nup35 on WT HIV-1 infection was direct, we sought to
75 examine the stability of other Nups after Nup35 depletion with siRNA. Knockdown of Nup35 did not
76 affect levels of other Nups present in the Nup93 subcomplex of which it is a member nor did it affect
77 the levels of TNPO3, Nup153, or Nup358 (Extended Data Fig. 3). These data suggested that the indirect
78 degradation of other Nups, including those known to interact with HIV-1, did not account for the
79 infection block. In addition, Nup153 continues to associate with the nuclear membrane in Nup35
80 knockdown cells as does the Nup93 subcomplex constituent Nup155, in the same relative orientation
81 to one another (Fig. 1b). Because N74D and P90A HIV-1 infection are unimpeded in Nup35-depleted
82 cells, coupled with the microscopy data, these results indicate transient Nup35 loss did not grossly
83 affect nuclear transport or NPC integrity.

84 We next tested three unique Nup35 siRNAs from the mixture used in the screen separately.
85 Consistent with the pooled siRNA results, two different siRNAs directed against Nup35, siRNA-2 and
86 siRNA-3, reduced WT HIV-1 infection approximately 10-fold (Fig. 1c) correlating with Nup35
87 depletion, whereas N74D HIV-1 and P90A HIV-1 were resistant to Nup35 depletion.

88 To test whether the reductions in HIV-1 infection were due to off-target effects, we performed
89 a functional rescue experiment (Fig. 1d). Nup35 siRNA-3 targets 3'-UTR sequence. Thus HeLa cells
90 were transduced either with LPCX empty vector or with LPCX vector expressing HA-tagged Nup35

91 that lacks the 3'-UTR and is therefore resistant to silencing by the siRNA. Western blot analysis
92 confirmed that siRNA knockdown reduced the endogenous level of Nup35 in both transduced cell
93 populations; however, ectopically expressed HA-Nup35 was unaffected by Nup35 siRNA treatment.
94 Consistent with prior results, cells depleted for endogenous Nup35 and transduced with the LPCX
95 empty vector were more than 10-fold less susceptible to HIV-1 infection. In contrast, Nup35 siRNA-
96 treated cells that expressed HA-Nup35 remained susceptible to WT HIV-1. N74D HIV-1 infection was
97 relatively consistent under the different Nup35 expression conditions. Notably, the small infection
98 decrease in MLV infection occurring after Nup35 knockdown was not offset in cells expressing
99 exogenous HA-Nup35 suggesting the effect on MLV could be indirect.

100 CRISPR/Cas9 gene-editing of *nup35* in HeLa cells confirmed the siRNA findings. While 5
101 different guide RNAs (gRNAs) were tested, only one targeting the third exon of *nup35* yielded cell
102 clones deficient of full-length Nup35. These clones uniformly expressed a truncated form of Nup35
103 that likely initiated from an in-frame methionine at coding residue position 62 of Nup35 within exon 3
104 based on the protein size and sequencing analysis (Fig. 1e and Extended Data Fig. 4a). The internally
105 initiated form of Nup35 may have permitted cell survival. These cells expressing the suspected N-
106 terminally truncated Nup35 had reduced susceptibility to HIV-1 infection but not N74D or P90A HIV-
107 1 infection (Fig. 1e). Consistent with siRNA knockdown results, knockout of Nup35 did not affect
108 levels of other Nups present in the Nup93 subcomplex nor did it affect the levels of TNPO3, Nup153,
109 or Nup358 (Extended Data Fig. 4b). Knockout cell clones for Nup153 (Extended Data Fig. 4c) were
110 also obtained and were similarly less permissive for HIV-1 infection relative to N74D or P90A HIV-1
111 infection (Fig. 1f). Although we were unable to detect a truncated form of Nup153 in the knockout cells
112 by western blot analysis, it is possible that a shorter form permitting survival was not recognized by
113 our antibodies.

114 Because N74D HIV-1 and P90A HIV-1 were insensitive to Nup35 depletion, we examined
115 other viruses with CA mutations that influence interaction with CPSF6 or CypA for infection of Nup35
116 knockdown cells. HIV-1 with N57A, Q63A/Q67A, K70A, or T107A mutations in CA are reduced in
117 sensitivity to CPSF6-mediated infection blocks and are similarly diminished in binding CPSF6 ¹².
118 Indeed, relative to WT HIV-1, these viruses were less sensitive to TNPO3 depletion which enables
119 CPSF6 inhibition of infection (Fig. 1g). While N57A, K70A, and N74D HIV-1 were similarly less
120 sensitive to Nup35 depletion, Q63A/Q67A and T107A HIV-1 resembled WT HIV-1 in infection
121 indicating that CPSF6-interaction does not predict Nup35 dependence.

122 In contrast to WT HIV-1, A92E HIV-1 and G94D HIV-1 replicate more efficiently in the
123 absence of CypA ^{13,14}. They however retain CypA as well as CPSF6 interaction sites. Similar to WT
124 HIV-1, these viruses were impaired for infection of Nup35 and TNPO3 knockdown cells (Fig. 1g). We
125 similarly tested simian immunodeficiency virus from macaques (SIV_{mac239}), which replicates
126 independent of CypA interaction, and feline immunodeficiency virus (FIV) which does not interact
127 with either CypA or CPSF6. SIV_{mac239}, which retains the conserved region necessary for CPSF6
128 interaction, was potently impaired by TNPO3 knockdown but exhibited reduced dependence on Nup35
129 (Fig. 1g). FIV was unaffected by either Nup35 or TNPO3 knockdown. Taken together, these results
130 demonstrate that HIV-1 CA determines sensitivity to Nup35 depletion, and the ability to interact with
131 CypA correlated with dependence on Nup35.

132

133 **CypA impairs HIV-1 infection in Nup35 knockdown cells.** To directly test whether CypA was
134 required for the HIV-1 dependence on Nup35, we treated Nup35-knockdown cells with CsA to inhibit
135 the CypA-CA interaction (Fig. 2a). WT HIV-1 infectivity was rescued to the level of control cells by
136 CsA treatment in Nup35-knockdown cells. In contrast, CsA had minor effects on WT HIV-1 infection

137 in either control cells or TNPO3-knockdown cells. N74D HIV-1 infection was previously demonstrated
138 to be sensitive to CsA treatment¹⁵, and it remained sensitive in Nup35- and TNPO3-knockdown cells.
139 As expected, the infectivity of HIV-1 with the CypA binding mutation P90A was not affected by CsA
140 treatment in any cell type. These data demonstrate that CsA restores HIV-1 replication following Nup35
141 knockdown. The effect was specific to HIV-1, as SIV_{mac239}, FIV, and MLV were not affected by CsA
142 in Nup35-depleted cells (Fig. 2a). Taken together, these data indicate that the block to HIV-1 infection
143 in Nup35-depleted cells is dependent on the virus binding CypA.

144 We sought to understand whether CypA affected WT HIV-1 dependence on Nup35 in the T cell
145 line, MT4, which are highly permissive to transduction with shRNA-expressing lentiviral vectors. Cells
146 were transduced with GFP-encoding shRNA vectors targeting Nup35 and then challenged with HIV-1
147 in the absence and presence of CsA (Fig. 2b). While knockdown of Nup35 specifically diminished WT
148 HIV-1 infection in 2 of 3 shRNA lines, CsA treatment restored WT HIV-1 infectivity to levels observed
149 in shRNA control cells treated with the drug. These data underline the pivotal role of CypA in
150 determining the HIV-1 sensitivity to Nup35 depletion.

151 Given the interrelation between CypA binding and HIV-1 sensitivity to Nup35 depletion, we
152 considered whether CsA-dependent viruses, A92E and G94D HIV-1, would remain sensitive to Nup35
153 depletion after CsA treatment. The CypA-dependent infection blocks that A92E and G94D HIV-1
154 exhibit in HeLa cells have been previously characterized as occurring at nuclear entry¹⁶. In cells
155 depleted of TNPO3, CsA treatment fails to fully restore the replication of these viruses indicating a
156 qualitatively different infection block (data not shown). In comparison, A92E and G94D HIV-1 were
157 also additionally sensitive to loss of Nup35 in cells (Fig. 2c). However in the presence of CsA, A92E
158 and G94D HIV-1 infectivity in Nup35 knockdown cells rises to the level of the mutant viruses in control
159 cells treated with CsA. Collectively, these data suggest the mechanisms underlying HIV-1 infection

160 inhibition in Nup35 knockdown cells may be similar to the infection blocks encountered by A92E and
161 G94D HIV-1 in normal cells in that CypA drives these viruses toward a nuclear entry pathway that
162 they are unable to exploit.

163

164 **HIV-1 infection in POM121 and Nup153 knockdown cells is affected by CypA.** The degree by
165 which CypA controlled HIV-1 sensitivity to Nup35 depletion led us to investigate whether HIV-1
166 interactions with POM121, which emerged from the same screen, and Nup153 were CypA-dependent.
167 Cells were either individually treated with siRNAs targeting Nup35, POM121, Nup153, and CypA, or
168 doubly treated with siRNAs targeting Nup35, POM121, and Nup153 in combination with siRNA
169 targeting CypA. Notably, depletion of CypA not only restored HIV-1 infection in Nup35 knockdown
170 cells but similarly restored infection in POM121 knockdown cells and partly restored infection in
171 Nup153 knockdown cells (Fig. 2d). A92E HIV-1 was also largely insensitive to the different Nup
172 knockdowns in cells where CypA was also depleted. By contrast, MLV infection was largely unaffected
173 in cells with single Nup knockdowns or Nup knockdowns in combination with CypA knockdowns
174 (Extended Data Fig. 5).

175

176 **Nup35 knockdown impairs HIV-1 nuclear entry.** To gain insight into the specific stage of HIV-1
177 infection at which Nup35 and CsA exert their effects, we performed time course experiments on
178 Nup35-knockdown HeLa cells that were treated with CsA at various times after exposure to virus. Up
179 to 12 hours post infection of Nup35-depleted cells, WT HIV-1 infectivity could be rescued by CsA
180 treatment; however, CsA completely lost rescue ability at 24 hours post-infection (Fig. 3a). Similarly,
181 CsA treatment also rescued the infectivity of CsA-dependent HIV-1 mutant A92E 12 hours post
182 infection, but could not rescue infectivity at 24 hours post infection. P90A HIV-1 and MLV infection

183 were not affected by CsA treatment at any time point, which is consistent with the inability of P90A
184 HIV-1 and MLV to bind CypA.

185 Given the early reversibility of the infection block and the alteration of the nuclear pore complex
186 by the various Nup knockdowns, we evaluated HIV-1 reverse transcription in cells depleted of Nup35
187 in the absence or presence of CsA (Extended Data Fig. 6 and Fig. 3b). We observed no difference in
188 the accumulation of early or late reverse transcription products in control cells vs. Nup35-knockdown
189 cells (Fig. 3b). Notably, both WT and A92E HIV-1 exhibited significantly reduced levels of 2-LTR
190 circle junction forms of viral DNA (vDNA) unless treated with CsA. Although 2-LTR circular vDNA
191 represents an abortive, noninfectious path, they form in the nucleus in the presence of DNA Ligase IV
192 and thus can be used to assess viral nuclear entry¹⁷. These data indicate that the Nup35 knockdown does
193 not impair reverse transcription but prevents HIV-1 nuclear entry in the presence of CypA.

194 We next tested whether CypA also regulated HIV-1 nuclear entry in cells depleted of POM121
195 or Nup153 using an assay based on CPSF6 distribution in HIV-1 infected cells. These cells were
196 depleted of Nup35, POM121, or Nup153 in the absence or presence of CsA. As before, WT and A92E
197 HIV-1 were sensitive to depletion of these Nups, and this infection reduction could be eliminated
198 through CsA treatment (Fig. 3c). By contrast, MLV infection was not affected by CsA in Nup35,
199 POM121, or Nup153 depleted cells (data not shown). Because CA interacts with CPSF6, soon after
200 infection, it can induce CPSF6 precipitates, especially in the nucleus where CPSF6 is abundant (Fig.
201 3d). In Nup35-knockdown cells, WT HIV-1 infection no longer causes CPSF6 precipitation in the
202 nucleus (Fig. 3e). Conversely, CsA treatment recovered CPSF6 aggregation following WT HIV-1
203 infection. As with the reverse-transcription staging data, these results indicate that restoration of HIV-
204 1 infection by CsA is linked to nuclear entry. Similar results were obtained with A92E HIV-1. Notably,
205 this virus did not induce nuclear aggregation of CPSF6 in control cells unless in the presence of CsA.

206 As expected, CsA treatment did not alter CPSF6 distribution in P90A HIV-1 infected control cells,
207 such that CPSF6 aggregates were observed in Nup35-knockdown cells in the absence of CsA. The
208 phenotypes of WT HIV-1, P90A HIV-1, and A92E HIV-1 were consistent in cells depleted of either
209 POM121 (Fig. 3f) or Nup153 (Fig. 3g). These results summarized in Fig. 3h indicate a pivotal role of
210 CypA in regulating HIV-1 nuclear entry in Nup35, Nup153, and POM121 knockdown cells.

211

212 **The choreography of HIV-1 interaction with host factors governing nuclear entry reveals a**
213 **regulatory switch.** Given the pivotal role that soluble factor binding appeared to exert on transport of
214 HIV-1 through the NPC, we considered whether differences in the subcellular localization of CPSF6
215 and CypA could predict their impact on this process. Although present throughout the cell, CypA is
216 enriched in cytoplasmic compartments, especially in proximity to the nuclear membrane in both HeLa
217 cells and T cell lines (Fig. 4a and Extended Data Fig. 7a-c). This distribution appears relatively stable
218 and is not grossly affected by Nup35-depletion (Extended Data Fig. 7d). CPSF6, consistent with its
219 role in pre-mRNA processing, is predominantly nuclear and distributed in a pattern quite distinct from
220 CypA and is also unaffected by Nup35 depletion (Extended Data Fig. 7e).

221 Indeed, in cells depleted of Nup35, POM121, or Nup153, only a partial restoration of WT HIV-
222 1 infection is observed after coordinate knockdown of CPSF6 (Fig. 4b), especially in Nup35 and
223 POM121 knockdown cells. HIV-1 infection in these cells can be fully restored after CsA treatment. In
224 comparison, P90A HIV-1 does not interact with CypA, and the infectivity of this virus is slightly
225 diminished in POM121 and Nup153 knockdown cells (Fig. 1a, Fig. 3c, and Fig. 4b). Whereas CsA
226 treatment, predictably, does little to affect P90A infection in the POM121 or Nup153 knockdown cells,
227 CPSF6 depletion potently enhances infection when in conjunction with either knockdown (Fig. 4b).

228 Although WT HIV-1 infection in Nup35 knockdown cells was elevated after CPSF6
229 knockdown, infection in Nup153 knockdown cells remained impaired even after CPSF6 knockdown,
230 suggesting distinct contributions of both Nups to HIV-1 nuclear entry. These observations were
231 confirmed and extended. While the effect of TNPO3 knockdown on HIV-1 infection could be
232 significantly ameliorated by CPSF6 depletion, and the effect of Nup35 knockdown on HIV-1 infection
233 could be partially offset by CPSF6 depletion, the reduction in HIV-1 infection by Nup153 knockdown
234 was unaffected by CPSF6 depletion (Fig. 4c). Notably, coordinate depletion of Nup35 and Nup153
235 potently impaired WT but not N74D HIV-1 infection.

236 We next asked whether CypA was also a key regulator of Nup155 use. Nup155 is a non-FG
237 Nup in the Nup93-subcomplex that we previously noted N74D HIV-1 to be more dependent for
238 infection than WT HIV-1². WT HIV-1 infection is diminished in cells depleted of Nup153 but retains
239 greater infectivity in Nup155 knockdown cells (Fig. 4d). Two distinct effects on infection are observed
240 when CypA is depleted in Nup153 and Nup155 knockdown cells. WT HIV-1 infection is increased in
241 the Nup153 knockdown cells as previously noted, and it is decreased in the Nup155 knockdown cells
242 (Fig. 4d). The infectivity of WT HIV-1 in the CypA plus Nup155 double knockdown cells is
243 comparable to N74D and P90A HIV-1 infection of the Nup155 cells. In the absence of CypA, it
244 becomes more dependent on Nup155. These data suggest the use of a secondary nuclear pathway for
245 HIV-1 when insertion into the Nup153 pathway is not possible.

246 Although our data suggest that the CA-dependence for HIV-1 use of Nup35 and POM121 is
247 linked to soluble host factor binding, it remains possible that these proteins also make direct contact
248 with HIV-1 despite their position within the NPC. We thus sought to examine whether fragments of
249 Nup35 or POM121 could interfere with HIV-1 in a CA-dependent manner. This approach has been
250 leveraged in mapping CA-interaction domains in CPSF6 and Nup153, specifically through fusion to

251 the N-terminal RING, B-box 2, and coiled-coil (RBCC) domains of rhesus TRIM5 alpha, substituting
252 the B30.2 (SPRY) domain with a region of interest ^{10,18}. Attempts to make rhTRIM5-Nup35 fusions
253 that were detectable in cell lines were unsuccessful. By contrast, rhTRIM5 fusions to the N-terminus
254 and C-terminus of POM121 were stable (Fig. 4e). Due to the potency of rhTRIM5-mediated restriction
255 and the sensitivity of WT, N74D, and P90A HIV-1 to POM121 depletion (Fig. 1a), we first tested
256 whether HIV-1 and MLV were differentially susceptible to infection in cells expressing the TRIM5-
257 POM121 fusion proteins (Fig. 4e). Whereas MLV displayed no apparent sensitivity to the different
258 TRIM5-POM121 proteins, HIV-1 (LAI Gag) was specifically inhibited by a TRIM5 fusion to the FG-
259 rich, C-terminal domain of POM121. Moreover, testing with HIV-1/MLV Gag chimeric viruses
260 revealed that HIV-1 CA was required for sensitivity to this protein. We extended this analysis to the
261 CA-mutant HIV-1 (NL4-3 Gag) isolates (Extended Data Fig. 8). Although HIV-1 CA governs
262 susceptibility to TRIM5-POM121-Cterm, the N74D and P90A mutations were insufficient to escape
263 restriction by the fusion protein.

264

265 **Discussion**

266

267 Here we demonstrate the dependence of HIV-1 on Nup35 and POM121 for nuclear entry. As
268 has been observed for Nup153 and Nup358, HIV-1 CA determines sensitivity to loss of Nup35 or
269 POM121. Extending prior work, soluble CA-interacting factors also regulate the necessity for the
270 nucleoporins. Unlike smaller cargo that interacts with cellular NTRs to achieve nuclear entry, we
271 propose that the HIV-1 core, comprised of multimeric CA in association with the viral nucleic acid and
272 enzymatic proteins, directly functions as a macromolecular NTR and negotiates multiple nucleoporin
273 interactions to achieve transfer through the NPC, through a pathway determined by soluble host factor
274 binding in the cytoplasm. Like cellular NTRs, CA selectively interacts with FG dipeptides^{10,12}. In
275 contrast to cellular NTRs, HIV-1 can enter the nucleus of metabolically inert cells without being
276 dependent on Ran-GTP regulation. Given that the host cell cytoplasm contains sensors to detect and
277 interfere with microbial pathogens, slipping into the nucleus quickly is advantageous to the virus.

278 Although only 24 kilodaltons in size as a monomer, the hundreds of CA molecules present in a
279 hexameric lattice composing the cytoplasmic HIV-1 core likely enable a distribution of protein-protein
280 interactions with host factors based on local concentrations and relative binding affinities. Human host
281 factors present in the cytoplasm known to interact with CA include CPSF6, CypA, and MxB. MxB is
282 thought to be enriched proximal to NPCs after interferon activation of cells¹⁹. By contrast, CPSF6 and
283 CypA are present at high steady levels, with CPSF6 predominantly enriched in the nucleus and CypA
284 abundant in the cytoplasm, especially in proximity to the nuclear membrane. HIV-1 has likely evolved
285 to exploit this concentration and distribution difference between CPSF6 and CypA in different
286 compartments to enhance access of NPCs, to ensure binding surfaces on CA were available for
287 interaction with specific Nups, and to utilize CPSF6 in the nucleus to access gene-rich regions of

288 chromatin. Prior studies have indicated that it is detrimental to the virus to engage these factors out of
289 order. Enrichment of CPSF6 in the cell cytoplasm blocks HIV-1 nuclear entry presumably by
290 preventing Nup interactions with the virus ^{2,20}.

291 The role of CypA in HIV-1 infection has been a long-standing puzzle. It was one of the first
292 host factors identified to interact with the Gag protein of an animal retrovirus ²¹. Although it had been
293 mapped to exert its effect on early replication steps prior to nuclear entry ^{22,23}, the precise mechanism
294 has been unclear. Studies with Nup153 and Nup358 suggested that CypA could affect subsequent
295 interactions with these Nups ^{5,24}, but to a limited extent. It is possible that the essential role that these
296 Nups play in the transport of all cargo limits analysis after they are depleted from cells. By contrast,
297 the effect of CypA on HIV-1 dependence of Nup35 or POM121 was dramatic. While HIV-1 was
298 blocked for infection in Nup35 or POM121 knockdown cells, after treatment with CsA, HIV-1 enters
299 the nucleus and infection is restored to levels of control cells. CypA-binding thus directed HIV-1 to a
300 nonproductive nuclear import pathway in the knockdown cells, possibly one that starts with Nup358
301 interaction. Thus, CypA might not only block premature CPSF6 interaction before docking at the pore,
302 its association with HIV-1 might facilitate a transfer of the core to Nup358, initiating a specific path of
303 passage through the NPC.

304 At this point, it is unknown whether Nup35 and POM121 make direct contact with HIV-1.
305 Nup35 is a member of the Nup93 subcomplex which encircles the central channel Nup62 subcomplex
306 comprised entirely of FG-Nups. Nup35 itself has three FG dipeptides, but it is unknown if these are
307 accessible to cargo. Given the demonstrated interaction of FG dipeptides in CPSF6 and Nup153 with
308 the N74 pocket of HIV-1 CA, it is tempting to speculate that this interface of HIV-1 negotiates a
309 multitude of interactions by the core during passage through the NPC. To a large extent, Nup35 and
310 POM121 knockdowns phenocopied Nup153 knockdowns with regards to CA-dependent effects on

311 HIV-1 infection. Similar to Nup153 and CPSF6, TRIM5 fusions to the FG-rich portion of POM121
312 inhibit HIV-1 infection. Recently, another group has also shown that a fragment of highly related
313 POM121C containing numerous FG-motifs was sufficient to impair HIV-1 infection ²⁵. While they did
314 not investigate whether POM121/POM121C could serve as a co-factor through knockdown/knockout
315 experiments, they argue that overexpression of truncated POM121C binds to a subunit of a cellular
316 NTR, Karyopherin subunit beta-1 (KPNB1), which possibly underlies a block to HIV-1 infection.
317 KPNB1 forms a complex with Importin-7 to mediate transport of cargo to the nucleus²⁶. KPNB1
318 interaction with POM121C is not unexpected given that NTRs interact with FG-Nups. More recent
319 studies have not supported a role for Importin-7 in HIV-1 infection ²⁷. Consistent with this finding, we
320 also did not observe removal of either KPNB1 or Importin-7 to effect HIV-1 infection (Extended Data
321 Fig. 9). Instead POM121 interaction with CA appears to support HIV-1 infection.

322 Notably, POM121 is known to interact with Sun1 ²⁸. Sun1 and Sun2 combine to form a linker
323 of nucleoskeleton and cytoskeleton (LINC) complexes which span the nuclear envelope ²⁹. Knockdown
324 or knockout of Sun2 reduces HIV-1 infection in human primary CD4+ T cells ³⁰ and THP-1 cells ³¹,
325 respectively. The CypA-dependence of Sun2 is unsettled. One study has shown that Sun2 promotes
326 CypA-dependent steps of HIV-1 replication in bone marrow-derived dendritic cells from *sun2*^{-/-}
327 mice³⁰, but other two studies have shown that there is no correlation between Sun2 and CypA in human
328 primary CD4+ T cells ³² and THP-1 cells ³¹. These differences could be due to cell context. Sun1 and
329 Sun2 form an inner nuclear membrane (INM) complex proximal to NPCs. It will be important to
330 determine the contribution of INM proteins with nucleoporins in influencing HIV-1 nuclear entry
331 pathway. How Sun2 interacts with HIV-1 is a subject of investigation.

332 Elucidating the precise choreography of HIV-1 interactions at the nuclear membrane and during
333 passage through the NPC is critical to understanding the successive contributions of each of these

334 factors. As illustrated in Figure 4c, the Nup35 and Nup153 blocks to HIV-1 infection were additive in
335 double-knockdown cells. One interpretation of these data is that the blocks are distinct and Nup35 loss
336 does not entirely prevent Nup153 use by HIV-1. It could be argued that the removal of both factors was
337 incomplete, so the effect of the double knockdown was increased potency in inhibiting the same
338 pathway. However, the genetics of both blocks are distinct. While there may be an overlapping need
339 for function by both Nup35 and Nup153 for optimal HIV-1 nuclear entry and infection, CPSF6,
340 potentially interacting with the virus within the NPC, creates a more stringent requirement on Nup35
341 (Fig. 4c) indicating functional differences between successive steps during nuclear import.

342 Indeed our prior work pointed to the existence of distinct routes of nuclear entry available to
343 HIV-1², which split along an axis of WT vs N74D HIV-1 CA exhibiting greater dependence on Nup153
344 vs Nup155, respectively. We find here that this switch in pathway use is governed in part by CypA.
345 N74D HIV-1 exhibits great sensitivity to the loss of CypA with an infection block in the cytoplasm¹⁵.
346 When Nup155 loss is coupled with CypA depletion in experiments presented here, a greater than 50-
347 fold decrease in N74D HIV-1 infectivity is observed. Moreover, WT HIV-1 also becomes more
348 dependent on the Nup155-import pathway in CypA-depleted cells. For WT HIV-1, a loss of CypA
349 binding diminishes access to the Nup153-import pathway. For N74D HIV-1, the N74D mutation of CA
350 precludes Nup153 utilization.

351 A competition between CypA and CPSF6 for binding to HIV-1 CA likely exists in the cell
352 cytoplasm given the overlap of binding sites. CypA is one of the most abundant proteins in the cell.
353 With the elevated concentration of CypA proximal the nuclear membrane, HIV-1 may have evolved to
354 bind it to prevent premature interaction with CPSF6 (Fig. 4f). CPSF6 aggregates are in fact observed
355 in the cytoplasm of cells infected with P90A HIV-1 (Fig. 3f-g). The affinity of full-length CPSF6
356 interaction with CA hexamers is currently unknown, although a peptide of the CA-binding site of

357 CPSF6 has an estimated affinity (K_D) of 50 micromolar⁴. CypA binding to the CA lattice at an affinity
358 (K_D) of approximately 12 micromolar³³. Thus it is unlikely that CPSF6 will exhibit preferential binding
359 to cores in areas of the cell where CypA is at higher concentration.

360 While both HIV-1 and SIVsm/mac/mne viruses retain CPSF6 binding interfaces, HIV-1
361 distinctly acquired a CypA interaction site in proximity to the CPSF6 binding interface. This potentially
362 reflects differences in the subcellular localization of CPSF6 in human vs some nonhuman primate cells.
363 If human cells have elevated CPSF6 levels outside the nucleus, this could impair HIV-1 interaction
364 with the NPC. As we observe here (e.g. Fig. 2a,b) and has been noted in past studies^{13,14}, CsA treatment
365 of some human cell targets results in diminished WT HIV-1 infection. Despite the potential danger of
366 cytoplasmic CPSF6 to the virus, CPSF6 has a positive effect on Nup35 and POM121 contribution to
367 HIV-1 infection (Fig. 4b,c). How to reconcile both CypA and CPSF6 effects on HIV-1 infection when
368 there might be competition for binding HIV-1 CA? We suggest a transition occurs after HIV-1
369 interaction with Nup358 at the cytoplasmic face of the NPC, previously hypothesized by Schaller et al
370⁵. Some CypA is likely displaced from the core by the Nup358-CypA related domain, which provides
371 CPSF6 and probably a subset of FG-Nups to subsequently interact with CA hexamers. This would
372 explain why the N74D HIV-1 infection pathway is different, resembling the entry route of other
373 lentiviruses, such as FIV^{2,24}. It fails to interact with CPSF6 within the NPC and uses distinct, currently
374 unidentified, surfaces on CA to negotiate potential nucleoporin interactions.

375 Despite WT and N74D HIV-1 exhibiting differences in their use of the NPC, there are common
376 themes. Removal of Nup62 subcomplex members (Nup54, Nup58, and Nup62), which are FG-Nups
377 that form a meshwork in the central channel of the pore restricting the flow of cargo larger than 5 nm
378 in diameter, generally elevated both WT and N74D HIV-1 infection. While loss of Nup54 and Nup58
379 elevated infection of WT HIV-1, N74D HIV-1, and P90A HIV-1, removal of Nup62 had a larger

380 positive effect on WT HIV-1 infection. Knockdown of Nup214, a FG-Nup that also is thought to
381 regulate the flow of cargo, also had a greater positive effect on WT HIV-1 infection. Collectively, WT
382 HIV-1 infection appears to be more broadly regulated by FG-Nups. Although we did not directly
383 investigate the role of CypA in facilitating Nup62 and Nup214 interaction, P90A HIV-1 infection was
384 2-3-fold lower than WT HIV-1 infection under knockdown conditions.

385 The ability of CypA/CPSF6 to regulate HIV-1 interactions with the NPC underscores the
386 vulnerability of the virus at this crucial replication step to MxB which also targets HIV-1 CA³⁴⁻³⁶. Both
387 N74D^{34,35} and P90A³⁴ HIV-1 show increased resistance to MxB antiviral function. Similarly, other
388 CypA and CPSF6 CA-binding mutants G89V and N57S, respectively, also exhibit MxB resistance³⁵.
389 MxB restriction has been associated with CypA function³⁶, and it is proposed that the block occurs at
390 the nuclear entry^{34,35}. The potential interplay of MxB with CypA or CPSF6, and whether it dysregulates
391 the temporal or spatial sequence of their interactions with CA, has not been examined at this point.

392 CA is intrinsic to critical replication steps in the cytoplasm, at the nuclear membrane, and within
393 the nucleus. Many of these actions are likely dependent on retention of a portion of the core, especially
394 given the N74 interface is accessed by factors when CA is in a hexameric configuration. The
395 coordination of different successive interactions among a limited subset of interfaces helps explain why
396 that this choreography is profoundly sensitive to disruption by small molecule inhibitors targeting CA,
397 such as PF-74 and BI-2^{37,38}.

398 Taken together, this study supports a model of HIV-1 CA co-opting CypA as a key factor in
399 preventing premature interactions in the cytoplasm by CPSF6 and presumably newly synthesized,
400 soluble nucleoporins until the virus reaches the nuclear membrane. Subsequently, the CA lattice
401 functions as a NTR which binds to different FG motifs present in FG-Nups or CPSF6 in the nuclear
402 channel enabling HIV-1 transport into the nucleus. The transposon Tfl is similarly hypothesized to use

403 an assembled Gag particle as a multimeric NTR, particularly in interacting with the FG-rich yeast
404 Nup124p during nuclear entry ³⁹. With regards to animal cell biology, as our and previous studies
405 indicate, HIV-1 provides an attractive model to interrogate the mechanism of nuclear entry by large
406 macromolecular entities.

407

408

409

410

411 **METHODS**

412

413 **Plasmids.** pNL4-3-Luc-E-R+ (HIV-1 vpr-positive, env-deleted, encoding firefly luciferase in place of
414 nef) or pHIV-RFP (HIV-1 vpr-negative, env-deleted, encoding RFP in place of nef) were used in
415 transfections with pL-VSV-G to generate VSV-G-pseudotyped HIV-1 vectors, and the MLV-based
416 retroviral vector pMX-RFP was used to generate Moloney-based virus in co-transfections with pJK3,
417 pL-VSV-G, and pCMV-Tat. Vectors for FIV⁴⁰ and SIV_{MAC239}⁴¹ have been described previously.

418 cDNA encoding human Nup35 was amplified from MGC Human Nup35 sequence-verified
419 cDNA (Accession number BC047029, GE Healthcare, Dharmacon), using primers that add an amino
420 terminal HA tag, and inserted into pLPCX-MCS between the EcoRI and NotI sites. All coding
421 sequences were confirmed by DNA sequencing.

422

423 **Cells and culture conditions.** HEK293T and HeLa cell lines were maintained in DMEM supplemented
424 with 10% FBS. H9, Jurkat, and MT4 cells were grown in RPMI 1640 plus 10% FBS. GHOST cells
425 were maintained in DMEM supplemented with 10% FBS, 500 ug/ml G418, 100 ug/ml hygromycin,
426 and 1 ug/ml puromycin.

427 To generate stable Nup35-expressing cell lines, HeLa cells were transduced with either wild-
428 type or mutant HA-tagged Nup35, a pLPCX-HA-Nup35 (cds) vector containing only the amino acid
429 coding sequence of the human Nup35 cDNA, which excludes the 3'-UTR, and the cells were selected
430 with 1 µg/ml puromycin (Millipore).

431

432 **siRNA screen.** To identify Nups requirements for HIV-1 infection, 32 human Nups were
433 systematically depleted using two different commercially available siRNA pools (Dharmacon; Sigma).

434 siRNAs were transfected into the HeLa cells at a 50 nM final concentration using RNAiMAX
435 (ThermoFisher Scientific), according to the manufacturer's instructions. After 2 days, the cells were
436 re-seeded into 24-well plates and infected with VSV-G-pseudotyped RFP reporter viruses at an MOI
437 of 0.5 in the presence of 5 ug/ml polybrene. After an additional 48 h incubation, cells were trypsinized
438 and the percentage of RFP-positive cells was scored by flow cytometry (FACSCalibur, BD
439 Biosciences). As a positive control, siRNA SMARTpool against TNPO3 (Dharmacon) was present on
440 each plate. To exclude false positives (e.g., due to toxicity), WT HIV-1, N74D HIV-1, and P90A HIV-
441 1 infections were performed in parallel to Moloney murine leukemia virus (MLV) infection on the
442 Nup-depleted cells. The first round of the screens was performed using siRNA from Sigma. Those
443 genes that showed a phenotype in the first round were confirmed by Dharmacon siRNA. Therefore,
444 two-thirds of the genes were screened using both Dharmacon on-target siRNA SMARTpool and Sigma
445 MISSION esiRNA. The screening was repeated in at least three independent experiments.

446

447 **Viral production.** To produce HIV-1 particles, pNL4-3-Luc-E-R+ or HIV-RFP (wild-type and capsid
448 mutants: N57A, Q63/67A, K70A, N74D, P90A, A92E, G94D, and T107A) was co-transfected with a
449 VSV-G expression vector at a ratio of 3:1 using Hilymax (Dojindo Molecular Technologies). The
450 medium was replaced after overnight incubation and viral supernatants were collected at 48 h post-
451 transfection. Viral supernatants were filtered and their infectivity was determined by using HeLa or
452 GHOST target cells.

453 MLV stocks were obtained by co-transfection of pLPCX, pJK3, pL-VSV-G, and pCMV-Tat at
454 a ratio of 4:2:1:0.3, respectively, with Hilymax. The medium was replaced one day after overnight
455 incubation and viruses were harvested at 48 h after transfection, passed through a 0.45- μ m filter, and
456 used directly to transduce target cells.

457

458 **Viral infection.** For single cycle infectivity assays, HeLa cells were plated in 24-well plates at 5×10^4
459 cells per well, and infected with single or serial-dilutions of VSV-G-pseudotyped HIV-1, SIV_{mac239},
460 FIV, or MLV in the presence of 5 ug/ml polybrene. In some experiments, 2.5 μ M cyclosporine A
461 (Bedford laboratories) was added to the culture media at the time of infection. At 48 h post-infection,
462 cells were washed, and the infection was analyzed by examining the percentage of RFP or GFP
463 expressing cells using flow cytometry (FACSCalibur, BD Biosciences), or by measuring firefly
464 luciferase activity (Promega).

465 For microscopy experiments, 2.5×10^4 HeLa cells were grown overnight on glass slides in a 24-
466 well plate. The next day, cells were infected with WT, P90A, or A92E pNL4-3-Luc-E-R+ at an MOI
467 of 50. At 12 h post-infection, cells were washed three times with PBS, fixed, permeabilized, and stained
468 with antibodies.

469 For analysis of HIV-1 reverse transcription products, HeLa cells were seeded at 2×10^5 cells per
470 well in 6-well plates and infected with VSV-G-pseudotyped wild-type HIV-1 or capsid mutant virus
471 (MOI of 1) in the presence or absence of 2.5 μ M cyclosporine A. For the negative control, a reverse
472 transcription inhibitor (EFV 150 nM) was added at the time of infection. The phenotype infection assay
473 was performed in parallel in 24-well plates.

474 **RNA interference.** Three ON-TARGETplus siRNAs directed against human Nup35 were purchased
475 from Dharmacon: siRNA 1, 5'-CUGCUGGUUCCUUCGGUUA-3'; siRNA 2, 5'-
476 AGAUAAAAGUGGCGCUCCA-3'; siRNA 3, 5'-AGUUAUUUCUACC GACACA-3'. HeLa cells
477 were plated at 1.5×10^5 cells per well in 6-well plates and transfected the next day with a final
478 concentration of 40 nM siRNA targeting Nup35 or non-targeting control siRNA (siCONTROL non-
479 targeting siRNA, Dharmacon), using RNAiMAX (ThermoFisher Scientific) according to the

480 manufacturer's instructions. To generate stable Nup35 knockdown cells, a lentiviral vector and the
481 following GIPZ Lentiviral (GE Healthcare, Dharmacon) shRNAs against hNup35 were used in this
482 study:

483 GIPZ Lentiviral Human Nup35 shRNA:

484 V3LHS_364366, TGTCTGTCAGAAATAACCT

485 V3LHS_380787, TATGAGCTGGTACAACCTGG

486 V3LHS_380788, TGGTTCAGATCCTAACGCG

487 Lentiviral vector stocks were produced by co-transfection of 293T cells with packaging plasmid Δ R8.2,
488 MISSION shRNA or GIPZ Lentiviral shRNA, and pL-VSV-G at a ratio of 1:1:0.5, the culture medium
489 was replaced at 12 h, and viral supernatants were collected and filtered at 48 h. HeLa cells were
490 transduced with the filtered supernatant and selected in 2 ug/ml puromycin.

491

492 **CRISPR-Cas9 knockout.**

493 To generate a CRISPR-Cas9 single guide RNA (sgRNA) expression vector, oligonucleotides were
494 annealed and inserted into the pX330 (Addgene plasmid #42230). The following sequences targeting
495 coding regions at the 5' end of the gene were used: 5'-GAAGGGCCACTAATTGATCG-3' (Nup35,
496 exon3) and 5'-GGACGCGGCGTTGCCACCAG-3' (Nup153, exon1). Non-targeting sgRNA was used
497 as control, 5'-CGCTTCCGCGGCCCGTTCAA-3'. HeLa knockout cells were generated by transiently
498 transfecting pX330 into the cells. Single clones were screened by western blotting and phenotype assay
499 and the candidate clones were verified by sequencing of genomic DNA using the following primers:

500 5'-cgGAATTCACATCTCCAAAGCCAGGAGTTA-3' and 5'-

501 taAAGCTTTGTATTTTATGTGTGGCCCAAG-3' (Nup35 target exon3), 5'-

502 cgGAATTCCTCTAAGGCCTCCGCCTCT-3' and 5'-
503 taAAGCTTGTCCCATACCTGATGCTGTTGT-3' (Nup153 target exon1).

504

505 **RNAi-resistant mutant generation and phenotype rescue.** To generate RNAi-resistant variants of
506 genes, the exogenous ORF transcript lacking the 3'-UTR sequence targeted by the siRNA was inserted
507 into LPCX vector. HeLa cells were then transduced with the filtered supernatant and the cells were
508 selected with 1 µg/ml puromycin (Millipore). For the rescue assay, puromycin-selected cells were
509 plated at 1.5×10^5 cells per well in 6-well plates and transfected the next day with siRNA (40 nM) against
510 human Nup35 3'-UTR region or control siRNA. Two days post-transfection, cells were re-seeded and
511 infected with the HIV-1 or MLV and analyzed by FACS 2 days post-infection.

512

513 **Quantification of HIV-1 reverse transcription products by real-time PCR.** HeLa cells were
514 transfected with siRNAs as described above. Two days later, cells were seeded at 2×10^5 cells per well
515 in 6-well plates and infected with VSV-G-pseudotyped HIV-1, or capsid mutant virus P90A HIV-1, or
516 A92E HIV-1. The virus was pretreated with 20 U ml^{-1} RNase-free DNase I (Roche) for 1 h at 37°C. At
517 2 h post-infection, the cells were washed once with PBS and fresh complete medium was added, cells
518 were then collected at 3, 6, 12 and 24 h after infection. Total DNA was extracted using the QIAamp
519 DNA Blood Mini Kit (Qiagen) and used for real-time PCR to specifically quantify HIV early reverse
520 transcription (RT) products, late RT products, and 2-LTR circle forms. The primer-probe sets and
521 conditions were used as previously described⁴². To normalize the amount of DNA in each PCR assay,
522 the following primer set was used to quantify the copy number of the cellular gene actin: forward, 5'-
523 TCACCCACACTGTGCCCATCTA CGA -3' and reverse 5'-
524 CAGCGGAACCGCTCATTGCCAATGG -3'. qPCR assays were performed using either Platinum

525 qPCR SuperMix-UDG (Invitrogen) for detecting HIV-1 reverse transcription or iQ SYBR Green
526 Supermix (Bio-Rad) for detecting actin.

527

528 **Western blotting.** Whole-cell extracts were prepared by lysing cells in RIPA buffer (Sigma-Aldrich),
529 equivalent protein content boiled in SDS sample buffer, resolved by Criterion Tris-HCl Precast Gels
530 (Bio-Rad), and blotted onto PVDF Blotting Membranes (GE Healthcare). Membranes were probed
531 with primary antibodies specific for human cyclophilin A (Abcam), CPSF6 (Novusbio), Importin 7
532 (Novusbio), KPNB1 (Novusbio), Nup35/53 (Abcam, Bethyl, GeneTex, or Novusbio), Nup93 (Abcam),
533 Nup153 (Abcam), Nup155 (Abcam), Nup188 (Novusbio), Nup205 (Novusbio), Nup358 (Abcam),
534 POM121 (GeneTex), TNPO3 (MyBioSource), HA (Sigma), and tubulin (Sigma) followed by
535 secondary HRP-conjugated anti-mouse (GE Healthcare) or anti-rabbit (GE Healthcare) antibodies and
536 detected using a Chemidoc XRS+ system (Bio-Rad).

537

538 **Immunofluorescence.** Cells were fixed with 4% paraformaldehyde (Boston Bioproducts) at room
539 temperature (RT) for 10 min, permeabilized with 0.2% Triton X-100 in PBS for 10 min, and then
540 blocked with 3% BSA for 30 min. Cells were incubated with primary antibodies at RT for 1 h, followed
541 by Alexa-Fluor-conjugated secondary antibodies and Hoechst 33342 (Thermo Fisher Scientific) at RT
542 for 30 min. Coverslips were mounted on glass slides with ProLong Gold antifade solutions (Molecular
543 Probes). Images were taken in Z-stacks using the Deltavision deconvolve microscope (GE Healthcare)
544 and deconvolved to remove out-of-focus light using the Softworks software (GE Healthcare). Primary
545 and secondary antibodies used for immunofluorescence were mouse anti-Cyclophilin A (Abcam),
546 rabbit anti-CPSF6 (Novusbio), mouse anti-Nup153 (Abcam), Rabbit anti-Nup155 (Abcam), mouse
547 anti-Nuclear Pore Complex (MAb 414, BioLegend), rabbit anti-Lamin B1 (Abcam), goat anti-rabbit

548 Alexa 488 (Molecular Probes), goat anti-mouse 488 (Molecular Probes), goat anti-mouse 546
549 (Molecular Probes), and goat anti-rabbit 555 (Molecular Probes).

550

551 **FIGURE LEGENDS**

552

553 **Figure 1. HIV-1 dependence on Nup35. a**, CA-dependent use of nucleoporins by HIV-1. HeLa cells
554 were transfected with a control siRNA or target siRNA (smart pool) for 48 h, then infected with m.o.i
555 of 0.5 of VSV-G-pseudotyped wild-type (WT) virus or virus harboring various CA mutants. After 48
556 h, infected RFP-positive cells were counted by FACS. Nups targeted by siRNAs are grouped by NPC
557 subcomplexes. TNPO3 siRNA-treated cells are included in all the screens as a positive control. WT
558 HIV-1 infection decreases of greater than 3-fold after knockdown of nucleoporins are encircled. Percent
559 relative infection is mean \pm s.d., from three independent experiments. MLV infection was also
560 measured at 48 h in parallel samples to monitor cell toxicity and specificity of HIV infection (Extended
561 Data Fig. 2). **b**, Distribution of Nup153 and Nup155 after Nup35 knockdown. Localization of Nup153
562 (red) and Nup155 (green) in HeLa cells by deconvolution microscopy after immunostaining. Lamin B1
563 is blue. A Z-section image is presented. **c**, different siRNAs targeting Nup35 impair HIV-1 infection.
564 HeLa cells were transfected with a control siRNA or three different siRNAs against Nup35 for 48 h,
565 then infected with VSV-G-pseudotyped WT HIV-1 vectors or virus harboring various CA mutants or
566 MLV. After 48 h, infected cells expressing fluorescent markers from viral vectors were enumerated by
567 FACS. Error bars show standard deviations of duplicates, representative of three independent
568 experiments. Western blot analysis confirmed Nup35 depletion by three different siRNAs. **d**, HeLa
569 cells stably expressing human HA-Nup35 lacking 3'-UTR or transduced with control vector (LPCX-
570 HA), were transfected with non-target (NT) siRNA or Nup35 siRNA targeting 3'-UTR region for 48

571 h, then infected with VSV-G-pseudotyped virus. Infected RFP-positive cells were counted by FACS.
572 Error bars show standard deviations of duplicates, representative of three independent experiments.
573 Western blot confirmed Nup35 restoration. **e, f**, HIV-1 CA determines Nup35 (**e**) or Nup153 (**f**)
574 dependence in HeLa knockout cell clones. Nup35 or Nup153 knockout HeLa cell clones were infected
575 by HIV-1 or MLV. Infected RFP-positive cells were counted by FACS 48 h after infection. Error bars
576 show standard deviations of duplicates, representative of three independent experiments. Western blots
577 show the knockout efficiency of Nup35 (**e**) or Nup153 (**f**). Empty, cell lines that only transiently co-
578 transfected pX330 with puromycin-expression vector, plasmid; NT, non-targeting control lines lacking
579 a gene-specific gRNA. **g**, HeLa cells were transfected with control siRNA or siRNAs against Nup35
580 or TNPO3 for 48 h, then infected with VSV-G-pseudotyped pNL4-3-Luc-E-R+ or virus harboring
581 various CA mutants, or various retroviruses as labeled. Firefly luciferase activity and GFP/RFP-
582 positive cells were monitored at 48h. Error bars show standard deviations of duplicates, representative
583 of two independent experiments. Western blotting confirmed knockdown efficiency.

584

585 **Figure 2. HIV-1 dependence on Nup35, Nup153, and POM121 is regulated by CypA. a**, HIV-1,
586 SIV, FIV, and MLV infection in the absence or presence of CsA in Nup35-depleted cells. HeLa cells
587 were transfected with control siRNA or siRNAs against Nup35 for 48 h, then infected with VSV-G-
588 pseudotyped viruses in the presence or absence of 2.5 μ M CsA. After 48 h, infected GFP/RFP-positive
589 cells were counted by FACS. Error bars show standard deviations of duplicates, representative of two
590 independent experiments. Western blotting confirmed knockdown efficiency. **b**, CsA restores HIV-1
591 infection in Nup35-depleted MT4 cells. MT4 cells were transduced with a control shRNA or three
592 different shRNAs against Nup35 then infected with VSV-G-pseudotyped WT or virus harboring
593 various CA mutants. Firefly luciferase activity was measured at 48 h. Error bars show standard

594 deviations of duplicates, representative of two independent experiments. Western blot analysis
595 confirmed Nup35 depletion by three different shRNAs. **c**, CsA restores A92E and G94D HIV-1
596 infectivity in Nup35 knockdown cells. HeLa cells were transfected with control siRNA or siRNA
597 against Nup35 for 48 h, then infected with increasing amounts of VSV-G-pseudotyped viruses in the
598 presence or absence of 2.5 μ M CsA, and firefly luciferase infectivity was measured at 48 h. Error bars
599 show standard deviations of duplicates, representative of three independent experiments. **d**, CypA
600 depletion by siRNA restores HIV-1 infectivity in Nup35 and POM121 knockdown HeLa cells. HeLa
601 cells were either single-knocked down (Nup35, POM121, Nup153, or CypA) or double-knocked down
602 (CypA in combination with Nup35, POM121 or Nup153) for 48 h. The cells were infected with VSV-
603 G-pseudotyped pNL4-3-Luc-E-R+ or CA mutants (P90A or A92E) and firefly luciferase infectivity
604 was measured at 48 h. MLV infection was also measured at 48 h in parallel samples to monitor cell
605 toxicity and specificity of HIV-1 infection (Extended Data Fig. 5). Error bars show standard deviations
606 of duplicates, representative of three independent experiments.

607

608 **Figure 3. Nup35 knockdown impairs HIV-1 nuclear entry. a**, CsA can restore HIV-1 infectivity in
609 Nup35 knockdown cells 12 h after virus challenge. HeLa cells were transfected with control siRNA or
610 siRNA against Nup35 for 48 h, then infected with VSV-G-pseudotyped viruses. CsA was added at
611 every 2 h until 12 h post-infection and at 24 h post-infection. Firefly luciferase activity was measured
612 at 48 h. Error bars show standard deviations of duplicates, representative of two independent
613 experiments. **b**, HIV-1 reverse transcription is blocked in Nup35 knockdown cells but 2-LTR circle
614 forms are not observed. HeLa cells were transfected with control or Nup35 siRNA for 48 h, then
615 infected with VSV-G-pseudotyped, DNase-treated NL4-3-Luc-E-R+ or CA mutants (P90A or A92E)
616 at an MOI of 1. The Cell DNA was extracted at 3, 6, 12, and 24 h after infection and used to detect

617 early (RU5) RT, late (2nd strand) RT, and 2-LTR circles (right). Error bars show standard deviations
618 of duplicates, representative of two independent experiments. The amount of DNA in each PCR assay
619 was normalized by actin. HIV-1 infection was also measured at 48 h in parallel samples to monitor
620 HIV-1 infectivity (Extended Data Fig. 6). **c**, CsA restores HIV-1 infection in Nup35, Nup153, and
621 POM121 knockdown HeLa cells. HeLa cells were transfected with control siRNA or siRNAs against
622 Nup35, POM121, or Nup153 for 48 h, then infected with infected with VSV-G-pseudotyped NL4-3-
623 Luc-E-R+ or CA mutants (P90A or A92E) viruses in the presence or absence of 2.5 μ M CsA. After 48
624 h, firefly luciferase infectivity was measured. Error bars show standard deviations of duplicates,
625 representative of three independent experiments. Western blotting confirmed knockdown efficiency. -
626 , no CsA; +, 2.5 μ M CsA. **d-g**, CPSF6 distribution of Nup35, POM121, and Nup153 knockdown cells
627 in the presence or absence of 2.5 μ M CsA. Cells were infected with VSV-G-pseudotyped NL4-3-Luc-
628 E-R+ or CA mutants (P90A or A92E) at an MOI of 50. After 12 h, cells were fixed and stained with
629 antibodies against CPSF6 and NPC. **h**, The number of cells with or without CPSF6 aggregates were
630 counted from (**d-g**).

631

632 **Figure. 4. Soluble factors regulate nucleoporin dependence.** **a**, CypA enrichment at the nuclear
633 periphery. Localization of CPSF6 (green) and CypA (red) in HeLa cells in a Z-section image from
634 deconvolution microscopy of immunostained HeLa cells. **b**, Partial restoration of HIV-1 infection in
635 Nup35 and POM121 knockdown cells after CPSF6 depletion. HeLa cells were either single-knocked
636 down (Nup35, POM121, Nup153, or CPSF6) or double-knocked down (CPSF6 with Nup35, POM121
637 or Nup153) for 48 h. The cells were infected with VSV-G-pseudotyped NL4-3-Luc-E-R+ or CA
638 mutants (N74D or P90A) viruses in the presence or absence of 2.5 μ M CsA, and firefly luciferase
639 infectivity and percentage of RFP-positive cells (MLV infection) were measured at 48 h. Error bars

640 show standard deviations of duplicates, representative of three independent experiments. Western
641 blotting confirmed knockdown efficiency. **c**, Distinct contributions of Nup35 and Nup153 to HIV-1
642 infection. HeLa cells were either single-knocked down (CPSF6, TNPO3, Nup35, or Nup153) or double-
643 knocked down (CPSF6 with TNPO3, Nup35, or Nup153; Nup35 with Nup153) for 48 h. The cells were
644 infected with VSV-G-pseudotyped HIV-RFP or CA mutant (N74D) viruses, and percentage of RFP-
645 positive cells were measured at 48 h. Error bars show standard deviations of duplicates, representative
646 of three independent experiments. Western blotting confirmed knockdown efficiency. **d**, CypA
647 determines HIV-1 dependency on Nup153 versus Nup155. HeLa cells were either single-knocked
648 down (CypA, Nup153, or Nup155) or double-knocked down (CypA with Nup153 or Nup155) for 48
649 h. The cells were infected with VSV-G-pseudotyped pNL4-3-Luc-E-R⁺ or CA mutants (N74D or
650 P90A) viruses, and firefly luciferase infectivity and percentage of RFP-positive cells (MLV infection)
651 were measured at 48 h. Error bars show standard deviations of duplicates, representative of three
652 independent experiments. Western blotting confirmed knockdown efficiency. **e**, TRIM5-POM121
653 inhibition of HIV-1 infection is CA-dependent. Top left, schematic representation of the rhesus TRIM5
654 RBCC (RING, B-box2, and coiled coil) domains (1-299) with the POM121 N-terminal fragments (1-
655 438) or C-terminal fragments (439-984). Top right, western blotting analysis of lysates from HeLa cells
656 expressing TRIM fusion proteins. Bottom left, schematic representation of the chimeric HIV/MLV
657 virus. Bottom right, HeLa cells stably transduced with HA-tagged rhTrim5a/POM121 fusion constructs
658 were infected with VSV-G-pseudotyped chimeric HIV/MLV virus, and firefly luciferase infectivity
659 was measured at 48 h. Error bars show standard deviations of duplicates, representative of three
660 independent experiments. Western blot analysis of HA-tagged rhTrim5a/POM121 fusion and control
661 constructs in stable HeLa cells (Extended Data Fig. 8). **f**, CypA determines the HIV-1 nuclear import
662 pathway. Premature CPSF6 interaction with HIV-1 impairs CA interaction with FG-Nups at the NPC.

663 CypA use by HIV-1, however, prevents CPSF6 access to the N74 pocket in CA until the virus docks
664 at the NPC. Subsequent CPSF6 binding may enhance release from the NPC. We propose that the HIV-
665 1 core, comprised of multimeric CA in association with the viral nucleic acid and enzymatic proteins,
666 directly functions as a NTR and exploits successive FG interactions to achieve transfer through the
667 NPC but these interactions are regulated in time and space by CypA and CPSF6.

668

669 **Acknowledgements**

670 We thank Szu-Wei Huang and KyeongEun Lee for intellectual contributions and scientific
671 discussions. We thank Eric Freed, Henry Levin, and Owen Pornillos for scientific suggestions, and
672 Eric Freed for MT4 cells. We thank Joseph Meyer from Scientific Publications, Graphics and
673 Media, Frederick National Laboratory for assistance with figure graphics. Dr. Xue was an NIH
674 Intramural AIDS Research Fellowship and NCI Sallie Rosen Kaplan Award recipient for women
675 scientists. This research was supported by the Intramural Research Program of the NIH,
676 Frederick National Lab, Center for Cancer Research. This work was also supported by NIH grants
677 5R01AI111809, 5DP1DA034990, and 1R01AI117839, to J.L. S.G.S. acknowledges support by NIH
678 grants P50 GM103368 and R01 AI120860. The content of this publication does not necessarily
679 reflect the views or policies of the Department of Health and Human Services, nor does mention
680 of trade names, commercial products, or organizations imply endorsement by the U.S.
681 Government.

682

683 **Extended Data Legends**

684

685 **Extended Data Figure 1: Depiction of the nuclear pore complex and HIV-1 CA hexamers. a,**
686 Major human NPC subcomplexes. FG-Nups hypothesized to interact with nuclear pore cargo based on
687 yeast or metazoan studies are emboldened. Image adapted from Ori *et al*⁶. **b and c,** Overlap of CypA
688 and CPSF6 binding regions in the HIV-1 capsid. Surface representation of the structure of two adjacent
689 hexamers from the cryo-EM model of capsid (CA) tubes (PDB ID: 3J34; yellow CA_{NTDS} and orange
690 CA_{CTDS}). **(b)** Binding site of a CPSF6 peptide (residues 313-327; magenta) based on crystal structures
691 PDB ID: 4U0A and 4WYM. **(c)** Binding site of CypA based on cryo-EM model PDB ID: 5FJB; blue.
692 The structures suggest that CypA bound to capsid may affect access of the full size CPSF6 to its binding
693 site.

694

695 **Extended Data Figure 2: Effect of nucleoporin knockdown on MLV infection.** HeLa cells were
696 transfected with a control siRNA or target siRNA (smart pool) for 48 h, then infected with m.o.i of 0.5
697 of VSV-G-pseudotyped MLV. After 48 h, infected RFP-positive cells were counted by FACS. This
698 analysis was performed in parallel the experiments shown in Fig. 1a. Percent relative infection is mean
699 \pm s.d., from three independent experiments. MLV, murine leukaemia virus.

700

701 **Extended Data Figure 3: Nup35 knockdown does not affect levels of other nucleoporins.** Nup35
702 knockdown does not affect levels of other proteins in the Nup93 subcomplex. Western blot analysis
703 with anti-Nup35 **(a)** or antibodies against Nup93 subcomplex proteins **(b)** using lysates from Nup35-
704 knockdown HeLa cells. **c,** Nup153 or Nup358 levels are unchanged in Nup35 knockdown cells.
705 Western blot analysis showing reactivity to anti-Nup35 or antibodies against TNPO3, Nup153, and
706 Nup358 using Nup35 or TNPO3 knockdown HeLa cell lysates.

707

708 **Extended Data Figure 4: Generation of knockout HeLa cells. a,** Genotyping of *nup35* knockout
709 clonal HeLa cell line. The protospacer adjacent motif (PAM) region is shown in orange, and the
710 CRISPR-Cas9 nuclease target sequence in blue. Gene editing events are indicated in red at the bottom
711 of the wild-type sequence. **b,** Expression of nucleoporins in Nup35 knockout cells. Western blot
712 analysis of Nup93 subcomplex proteins as well as TNPO3, Nup153, and Nup358 from Nup35 knockout
713 HeLa cell lysates. **c,** Genotyping of *nup153* knockout clonal HeLa cell line. The PAM region is shown
714 in orange, and the CRISPR-Cas9 nuclease target sequence in blue. Gene editing events are indicated in
715 red at the bottom of the wild-type sequence.

716

717 **Extended Data Figure 5: Knockdowns of CypA-dependent nucleoporins have minor effects on**
718 **MLV infection.** HeLa cells were either single-knocked down (Nup35, POM121, Nup153, or CypA) or
719 double-knocked down (CypA with FG-Nups, Nup35, POM121 or Nup153) for 48 h. The cells were
720 infected with VSV-G-pseudotyped MLV and infected RFP-positive cells were counted by FACS. This
721 analysis was performed in parallel to the experiments shown in Fig. 2d. Error bars show standard
722 deviations of duplicates, representative of three independent experiments. Western blotting confirmed
723 knockdown efficiency.

724

725 **Extended Data Figure 6: Nup35 knockdown impairs HIV-1 nuclear entry.** HeLa cells were
726 transfected with control siRNA or siRNA against Nup35 for 48 h, then infected with increasing
727 amounts of VSV-G-pseudotyped viruses in the presence or absence of 2.5 μ M CsA. Firefly luciferase
728 activity and percentage of RFP-positive cells (MLV infection) were measured at 48 h. Western blotting
729 confirmed knockdown efficiency. This analysis was performed in parallel the experiments shown in
730 Fig. 3b.

731

732 **Extended Data Figure 7: Subcellular distribution of soluble factors. a-c**, Distribution of CPSF6
733 and CypA in MT4 (a), Jurkat (b), and H9 (c) cells. Localization of CPSF6 (green), CypA (red), and
734 lamin B1 (blue) in a Z-section of immunostained cells imaged by deconvolution microscopy. **d**, Nup35
735 depletion does not alter CypA subcellular localization. Localization of CypA (green) and lamin B1
736 (red) in a Z-section of immunostained HeLa cells imaged by deconvolution microscopy. **e**, Nup35
737 depletion does not alter CPSF6 subcellular localization. Localization of CPSF6 (green) and lamin B1
738 (red) in a Z-section of immunostained HeLa cells imaged by deconvolution microscopy.

739

740 **Extended Data Figure 8: C-terminal domain of POM121 is required for restriction of HIV-1**
741 **infection.** HeLa cells stably transduced with HA-tagged rhTrim5a/POM121 fusion constructs were
742 infected with VSV-G-pseudotyped HIV-RFP or CA mutants (N74D or P90A) viruses, and percentage
743 of RFP-positive cells was measured at 48 h.

744

745 **Extended Data Figure 9: Efficient HIV-1 infection in cells depleted of KPNB1 or Importin-7.**
746 HeLa cells were transfected with control siRNA or siRNAs against TNPO3, POM121, KPNB1, or
747 Importin 7 for 48 h, then infected with VSV-G-pseudotyped HIV-RFP or MX-RFP. After 48 h, RFP-
748 positive cells were enumerated by FACS. Error bars show standard deviations of duplicates,
749 representative of two independent experiments. Western blotting confirmed knockdown efficiency.

750

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- 866

Figure 1

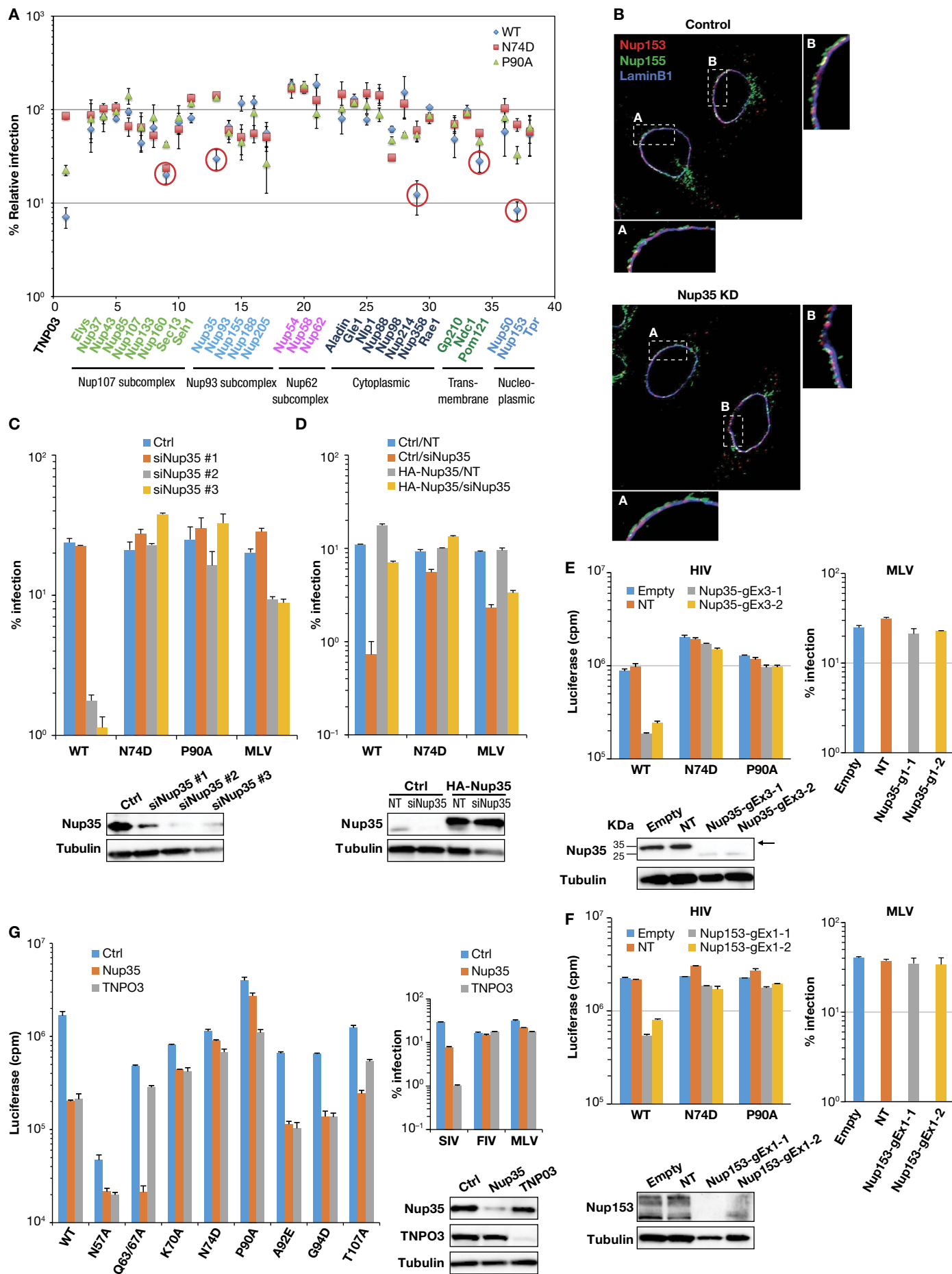


Figure 2

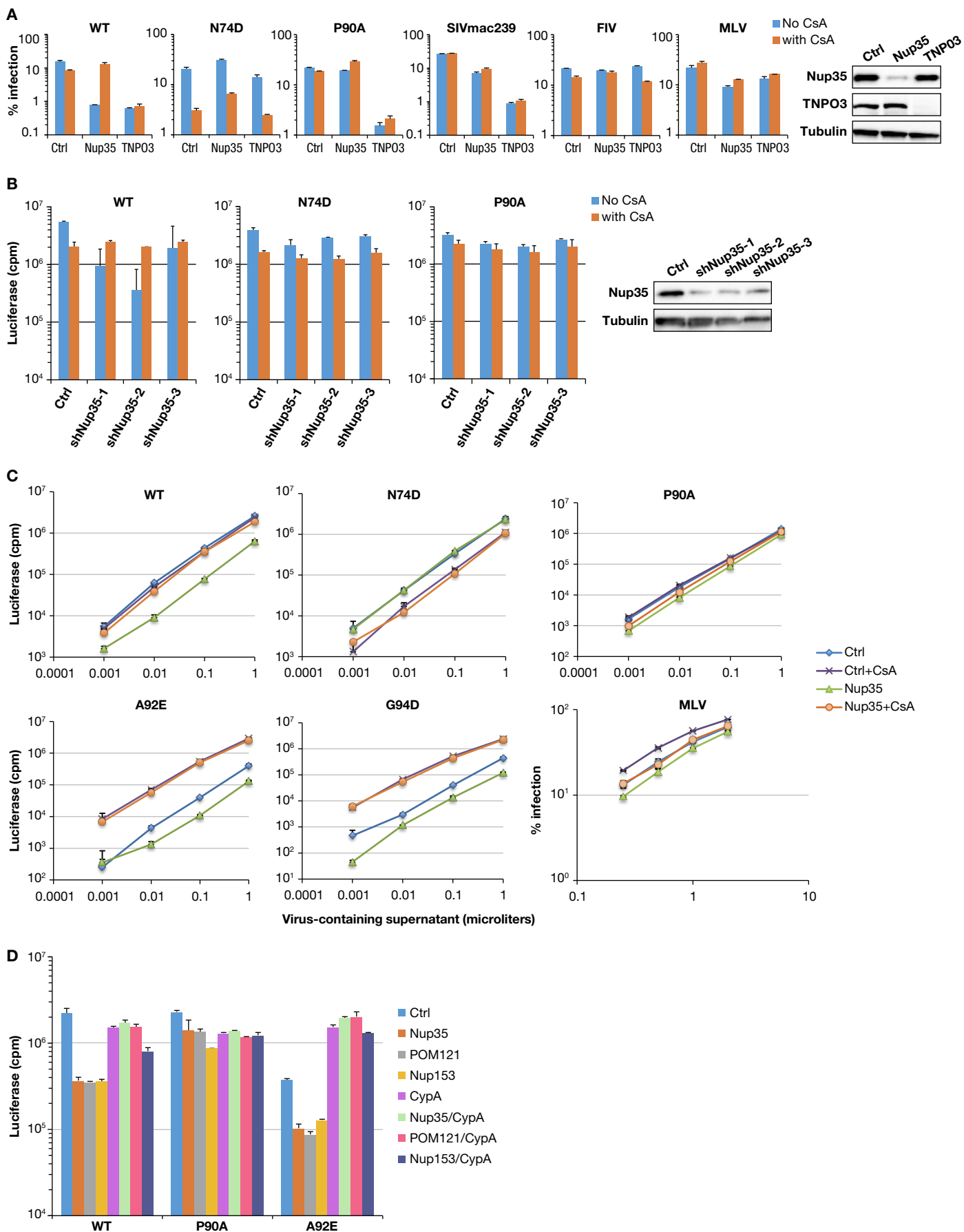


Figure 3

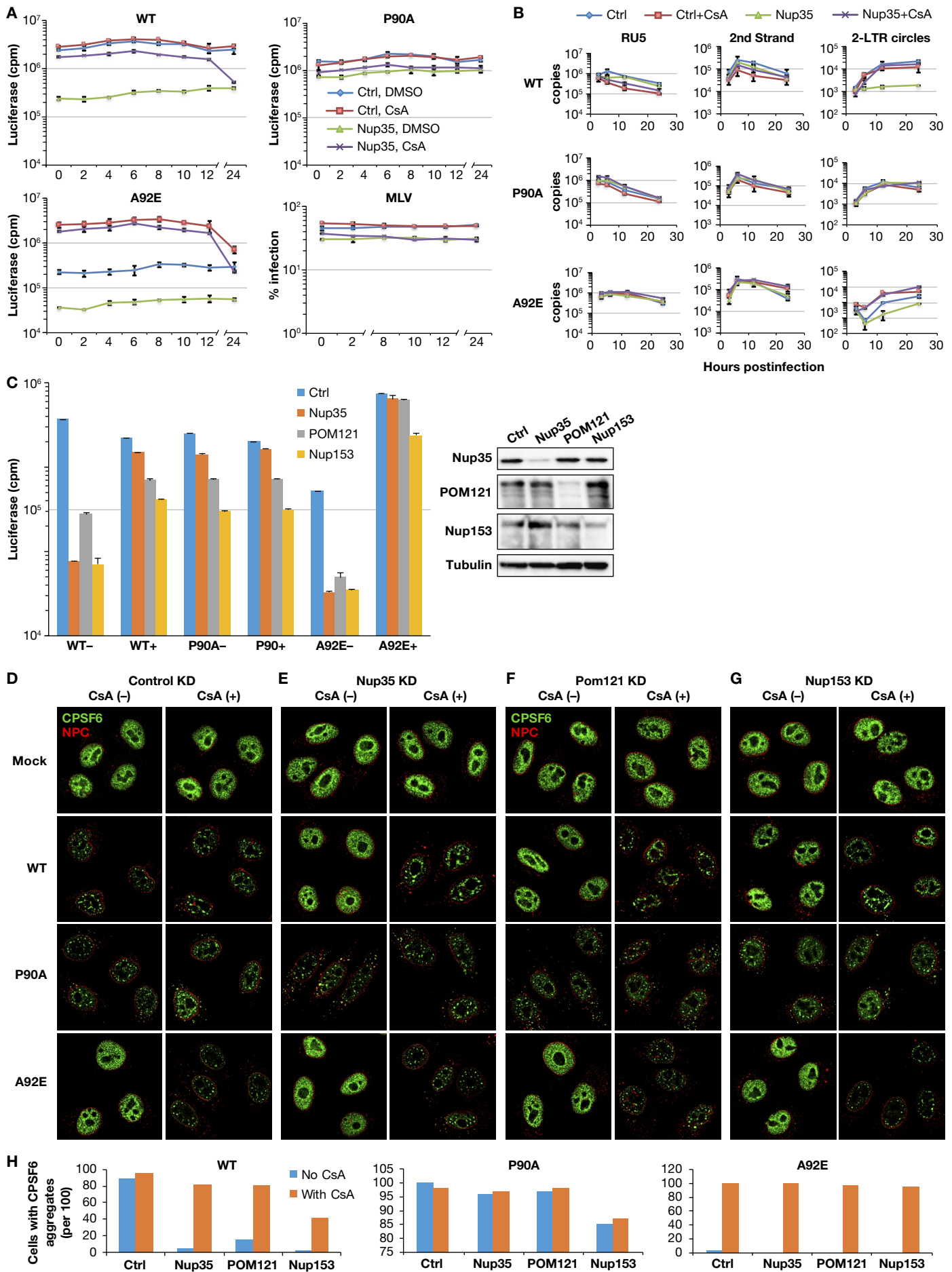


Figure 4

