Retroviral infection involves the reverse transcription of the viral RNA genome into DNA, which is subsequently integrated into the host cell genome. Human immunodeficiency virus type 1 (HIV-1) and other lentiviruses mediate the infection of non-dividing cells through the ability of the capsid protein to engage the cellular nuclear import pathways of the target cell and mediate their nuclear translocation through components of the nuclear pore complex. Although recent studies have observed the presence of the capsid protein in the nucleus during infection, reverse transcription and disassembly of the viral core have conventionally been considered to be cytoplasmic events. Here, we use an inducible nuclear pore complex blockade to monitor the kinetics of HIV-1 nuclear import and define the biochemical staging of these steps of infection. Surprisingly, we observe that nuclear import occurs with relatively rapid kinetics and precedes the completion of reverse transcription in target cells, demonstrating that reverse transcription is completed in the nucleus. We also observe that HIV-1 remains susceptible to the capsid-destabilizing compound PF74 following nuclear import, revealing that uncoating is completed in the nucleus. Additionally, we observe that certain capsid mutants are insensitive to a Nup62-mediated nuclear pore complex blockade in cells that potently block infection by wild-type capsid, demonstrating that HIV-1 can use distinct nuclear import pathways during infection. These studies collectively define the spatio-temporal staging of critical steps of HIV-1 infection and provide an experimental system to separate and thereby define the cytoplasmic and nuclear stages of infection by other viruses.

Extensive examinations of the spatio-temporal staging of the human immunodeficiency virus type 1 (HIV-1) life cycle have supported a model where HIV-1 completes reverse transcription and substantial capsid disassembly in the cytoplasm or at the nuclear pore complex (NPC). The understanding of the spatio-temporal staging of these events has relied on both biochemical and imaging approaches. These studies have largely relied on monitoring indirect measures of nuclear import, such as the formation of 2-long terminal repeat (2-LTR) circles or the behaviour of populations of viral particles that may or may not lead to productive infection.

To overcome these limitations and test this model of infection, we employed an inducible NPC blockade that allowed us to determine the spatio-temporal staging of reverse transcription, capsid disassembly and the kinetics of nuclear import of infectious HIV-1 viral particles after a synchronized infection. An inducible NPC blockade was achieved by transducing HIV-1 target cells with a lentiviral vector expressing Nup62 fused to a drug-inducible dimerization domain B (DmrB) and two copies of enhanced green fluorescent protein (eGFP) (Fig. 1a). Nup62 is a nucleoporin known to localize to the central pore of the NPC; a previous study demonstrated that an Nup62-DmrB-eGFP (Nup62DG) fusion can allow for inducible ciliary and NPC blockade after the addition of a rapamycin analogue homodimerizing drug (HD) that induces homodimerization of the DmrB domain. To monitor the efficacy of the Nup62DG construct to block active NPC transport, we monitored the nuclear translocation of oestrogen receptor-α (ERα) induced by the addition of oestradiol (E2). Consistent with previous observations, expression of this Nup62DG construct effectively prevented nuclear import of ERα in the presence of the HD homodimerizing compound and E2 (Fig. 1b and Extended Data Fig. 1b). No difference in the rate of nuclear accumulation of ERα was observed after E2 treatment in the absence of HD treatment (Extended Data Fig. 1a,c), demonstrating that Nup62DG expression does not perturb nuclear import when NPC blockade is not induced. After infection with the HIV-1 strain R7ΔEnvmCherry pseudotyped with vesicular stomatitis virus (VSV) glycoproteins, cells stably expressing this construct were potently inhibited by addition of HD, while infection was not affected by expression of the construct or addition of HD alone (Fig. 1c and Supplementary Fig. 1). NPC blockade did not influence reverse transcription but inhibited the formation of 2-LTR circles, a surrogate marker of nuclear import (Fig. 1d), which is consistent with a specific block of HIV-1 nuclear import.

Visualization of HIV-1 particles in cells where nuclear import was blocked revealed lower p24 signal inside the nucleus and accumulation of HIV-1 particles at or near the nuclear membrane, often in complex with the Nup62DG construct (Fig. 1e–g). In the absence of drug treatment, we also observed colocalization of HIV-1 with Nup62DG in the cytoplasm (Fig. 1e). This was not specific to the Nup62DG construct, since we observed similar relocation of endogenous Nup62 during infection and colocalization with HIV-1 following infection (Extended Data Fig. 3), while Nup62 was almost exclusively localized to the NPC in the absence of infection. Live cell imaging revealed HIV-1 trafficking in complex with Nup62DG away from the nuclear envelope after NPC blockade (Extended Data Fig. 4 and Supplementary Video 1). These observations are similar to our previous observations of Nup358 relocation during HIV-1 infection and demonstrates that HIV-1 infection induces dynamic relocation of numerous NPC components during infection.
**Fig. 1 | Artificial NPC blockade inhibits HIV-1 infection at the nuclear entry stage.**

**a.** Schematics of the Nup62 construct fused to DmrB and two copies of eGFP (Nup62DG) used to block active nuclear pore transport after the addition of B/B HD. **b.** HeLa cells were stably expressing the Nup62DG construct and were transfected with ER-α fused to mCherry. Twenty-four hours post-transfection, cells were treated with E2 for 30 min in the presence or absence of HD. The efficiency of nuclear pore blockade was quantified by counting cells having either a nuclear ER-α signal (less efficient) or both nuclear and cytoplasmic ER-α signals (efficient nuclear pore block). The data shown are representative of three independent experiments. **c.** Mock or Nup62DG-transduced HeLa, THP1-differentiated macrophages, CEM and SupT1 T cells were synchronously infected with VSV-G pseudotyped R7ΔEnvmCherry in the presence or absence of HD drug for the first 24 h of infection. The HD drug was removed after 24 h, replaced with normal media and infection was assessed 48 h post-synchronization by measuring the percentage of mCherry+ cells. The normalized and averaged data (± s.e.m.) from three independent experiments are shown. **d.** RT-qPCR quantification of reverse transcription and 2-LTR circles in cells expressing Nup62DG, 24 h after HIV-1 infection. Depicted is the mean of biological triplicates (± s.d.). Data shown are representative of three independent experiments. **e.** HeLa and THP1-differentiated macrophages that stably express the Nup62DG construct were synchronously infected with VSV-G-R7ΔEnvmCherry. Cells were treated with the HD drug for 4 h, then fixed and stained for HIV-1 capsid protein; p24 (red) and DAPI (blue) for the cell nucleus. Colocalization between Nup62DG with p24 (boxed region) is shown by the arrows. **f, g.** We employed a quantification process as described in the Methods and Extended Data Fig. 2, to detect perinuclear and nuclear p24 protein levels in the HeLa- (f) and THP1-differentiated (g) macrophages described in e. Twenty or more cells were analysed in each experiment. Data were averaged (± s.e.m.) from three independent experiments. Statistical significance was assessed using a two-way ANOVA and Bonferroni post-test. $P < 0.05$ was considered significant.
We next used this selective NPC blockade to determine HIV-1 nuclear import kinetics (NIK) in various target cells. After synchronized infection, NPC blockade was induced at various times after infection to determine the rate at which the viral inoculum became insensitive to NPC blockade (Fig. 2a). Notably, HIV-1 became resistant to NPC blockade much more quickly in monocytic and T-cell
lines, monocyte-derived macrophages (MDMs) and CD4+ primary T cells than HeLa cells (Fig. 2), with approximately half of the inoculum becoming resistant to NPC blockade approximately 3.5 h after synchronized infection in these cell types (Fig. 2c,d), compared to approximately 7 h in HeLa cells (Fig. 2b).

The rate at which HIV-1 became insensitive to NPC blockade suggested that HIV-1 nuclear import may occur before the completion of reverse transcription, which is generally considered to be complete before nuclear import. Completion of reverse transcription occurs with much slower kinetics than the kinetics of nuclear import observed in cells, particularly in macrophages26. Therefore, we examined the rate at which HIV-1 became insensitive to NPC blockade and reverse transcription inhibition in MDMs and THP-1 differentiated macrophages to test the hypothesis that nuclear import precedes completion of reverse transcription. These experiments revealed that the virus remains sensitive to reverse transcription inhibition for hours after the inoculum has become insensitive to the NPC blockade (Fig. 3a,b and Extended Data Fig. 5). CD4+ T cells also remained sensitive to reverse transcription inhibition for hours after the viral inoculum became insensitive to NPC blockade (Fig. 3c). Although less pronounced, significant differences were also noted in CEM and SupT1 T-cell lines (Extended Data Fig. 6a,b). Similar results obtained after infection with HIV-1 pseudotyped with CXCR4 tropic (HXB2) HIV-1 glycoproteins (Extended Data Fig. 6c). Collectively, these data suggest that infectious HIV-1 virions enter the nucleus many hours before reverse transcription is complete. To validate this surprising observation, we ordered hybridization probes specific for each DNA strand formed during reverse transcription (Supplementary Fig. 2). Consistent with the kinetics of nuclear import and reverse transcription sensitivity observed earlier, we note that the puncta positive for both DNA strands did not appear until 9 h after infection and that these puncta were exclusively nuclear in THP1-differentiated macrophages and MDMs (Fig. 3d and Extended Data Fig. 7a,b). These data collectively demonstrate that the viral ribonucleoprotein complex enters the nucleus before the completion of reverse transcription.

Previous studies exhibited a lack of consensus regarding the role of reverse transcription in HIV-1 nuclear import. While some studies showed that reverse transcription promotes cytoplasmic uncoating of the viral core before nuclear import24–28, other studies have suggested that HIV-1 nuclear import can occur in the absence of reverse transcription15. To determine the degree to which cytoplasmic reverse transcription is required for functional nuclear import, we examined the NIK of cells infected in the presence of reverse transcription inhibitor nevirapine (NVP) for 6 h. Although NVP treatment for 6 h did not impact infection measured at 48 h in the absence of NPC blockade, we observed that the viral inoculum in the NVP-treated infection was unable to bypass the NPC blockade until NVP was washed out (Fig. 3e,f and Extended Data Fig. 8a). After withdrawal of NVP, nuclear import of the inoculum rapidly recovered and approached the kinetics of the nuclear import of untreated infections at later time points. Consistent with these observations, we also observed that NVP treatment prevented the relocalization of NPC components to the cytoplasm (Extended Data Fig. 8b–f). However, we also observed that reverse transcription inhibition reduces but does not eliminate the accumulation of p24 capsid in the nucleus (Extended Data Fig. 9a,b). These results collectively demonstrate that although reverse transcription can be completed in the nucleus, some amount of cytoplasmic reverse transcription is necessary to facilitate productive nuclear import of the virus. The observation that reverse transcription is required to induce the relocalization of NPC components during infection is also consistent with a role for reverse transcription HIV-1 nuclear import.

One reason that reverse transcription has generally been assumed to be completed in the cytoplasm is that the strand transfer events that occur during reverse transcription probably require the constrained environment of the capsid core to prevent the premature diffusion of the reverse transcriptase enzyme away from the viral genome. However, most studies of core disassembly or uncoating suggest that this process is completed before the nuclear import of the viral ribonucleoprotein complex13,29. Although some capsid protein has been shown to remain with the viral ribonucleoprotein complex in the nucleus23–25, it has previously been difficult to demonstrate that this capsid plays a functional role in infection subsequent to driving the final stages of nuclear import. Therefore, we used PF74, which is known to bind to the interface between capsid monomers in assembled capsid and inhibit infection27–32. PF74 susceptibility is therefore dependent on the assembled capsid; continued PF74 susceptibility after infection reveals a continued functional role for the assembled capsid at subsequent steps of infection. Therefore, we used PF74 in the context of the NIK assay to determine if the assembled capsid mediates a functional role in infection following nuclear import. As was observed with reverse transcription inhibitors, these experiments revealed that the viral inoculum remains sensitive to PF74 inhibition for hours after the virus becomes insensitive to NPC blockade in all target cells examined (Fig. 4). While this effect was more pronounced in MDMs, primary CD4+ T cells and THP1 cells (Fig. 4a,b,c), we observed significant differences in NPC blockade and PF74 sensitivity in SupT1 and CEM cells (Fig. 4c,d), revealing that the assembled capsid mediates a functional step in infection subsequent to capsid-mediated import of HIV-1.

Finally, since capsid is known to mediate the nuclear import of HIV-1 and mutations in capsid are reported to influence the nuclear import pathway used during infection24, we next wanted to determine the degree to which capsid mutants influence the NIK of HIV-1. However, infection with HIV-1 containing the N74D capsid, which is defective in binding to CPSF6 and Nup153 (refs. 34,35); the P90A capsid, which cannot bind cyclophilin A, exhibited weaker susceptibility to Nup62-mediated NPC blockade compared to wild-type virus in the same target cells (Extended Data Fig. 10a,b). This suggests that these mutants, particularly the P90A mutant, enter the nucleus through distinct NPCs, which is consistent with a recently proposed model of NPC heterogeneity influencing HIV-1 infection and inhibition by the restriction factor MX2 (ref. 36).

In this study, we used an inducible NPC blockade to monitor the rate at which a synchronized HIV-1 infection becomes insensitive to NPC blockade. The ability to measure nuclear import under these conditions by monitoring HIV-1 infection provides substantial advantages over comparable imaging or biochemical approaches, thus allowing valuable insights into the spatio-temporal staging of key aspects of HIV-1 infection, including completion of reverse transcription and core disassembly. These results reveal that nuclear import occurs with faster kinetics than previously appreciated using 2-LTR circles to measure HIV-1 nuclear import. Nuclear import also precedes other events of the life cycle, including the completion of reverse transcription and uncoating, which have previously been considered to be cytoplasmic steps of HIV-1 infection. The observation that HIV-1 remains susceptible to PF74 following nuclear import reveals that the assembled capsid mediates a critical nuclear step of infection that is independent of NPC translocation, as suggested by biochemical studies27,28 and consistent with another study published online while this article was in revision36.

The relative insensitivity of the N74D and P90A capsid mutants to Nup62 dimerization was another unexpected outcome in our study. While other studies support the notion that capsid influences aspects of nuclear import and subsequent integration site selection1,4,7, the selective blocking of individual capsid mutants more specifically suggests that these viruses enter the nucleus through distinct nuclear pores that differ in their constituent nucleoporin composition. This possibility that nuclear pores are heterogeneous in nucleoporin composition and mediate the import of different
Fig. 3 | HIV-1 reverse transcription is required for efficient nuclear import and is completed in the nucleus of the infected cell. a–c, THP1-differentiated macrophages (a), primary human macrophages (b) and primary CD4⁺ T cells (c) expressing the Nup62DG construct were infected with VSV-G-R7ΔEnvmCherry and NIK was monitored by adding HD (red) as described in Fig. 2. To monitor replication kinetics, the HIV-1 reverse transcriptase inhibitor NVP (5µM) was added at different times post-infection as shown (green) and was washed off at 24h. Infectivity was measured as described in Fig. 2. The bar graphs represent data from a single experiment and the line graphs represent normalized and averaged data (± s.e.m.) as described in Fig. 2 from three independent experiments. d, The presence of HIV-1 nucleic acids in the nucleus of THP1-differentiated macrophages and primary human macrophages was monitored. Cells were infected with VSV-G-R7ΔEnvmCherry and were fixed at different time points post-synchronized infection. Cells were treated with RNase A and stained for (−) vDNA and (+) vDNA using specific sense and antisense probes. The graph depicts the average number of (−) vDNA positive for (+) vDNA inside the nucleus from three independent experiments. Twenty or more cells were analysed in each experiment. The error bars represent the s.e.m. from three independent experiments. e, f, THP1-differentiated macrophages (e) and primary human macrophages (f) expressing the Nup62DG construct were infected with VSV-G-R7ΔEnvmCherry and NIK was monitored after adding HD alone (red) or in the presence of NVP treatment for the first 6 h (blue) after synchronized infection. The bar graphs represent data from a single experiment and the line graphs represent the normalized and averaged data (± s.e.m.) as described in Fig. 2 from three independent experiments. Statistical significance was assessed using a two-way ANOVA and Bonferroni post-test. P < 0.05 was considered significant. NT, no treatment.

cargoes within individual cells is consistent with another recent study of HIV-1 nuclear import①, but otherwise remains largely unappreciated and poorly characterized. Inducible dimerization of other NPC components may allow the composition of these alternative NPCs to be more clearly defined and allow the cytoplasmic and nuclear stages of HIV-1 infection, as well as infection of other viruses that enter the nucleus during infection, to be clearly established.

The observation that functional nuclear import (the nuclear import of infectious HIV-1) is inhibited when reverse transcription is inhibited is consistent with previous studies demonstrating that reverse transcription can induce HIV-1 uncoating in cells⑥,⑦ and in vitro⑧. However, the observation that reverse transcription inhibition does not similarly reduce the amount of p24 in the nucleus (Extended Data Fig. 9) is consistent with other studies suggesting that nuclear import of HIV-1 can occur independently of reverse transcription⑥,⑨. This observation, taken together with the observation that Nup62 blockade does not completely abolish infection (Fig. 1c) and that some capsid mutants can enter the nucleus using distinct nuclear import pathways (Extended Data Fig. 10) suggest that differences in previous studies may be explained by the existence of multiple nuclear import pathways used by HIV-1. Our data suggest that reverse transcription promotes the use of
the primary import pathway used during infection, which our data suggest is more sensitive to Nup62 blockade than the alternative import pathways used by mutants such as P90A and N64D. The observation that p24 accumulation in the nucleus is not substantially reduced after inhibition of reverse transcription, but does not lead to infection, probably reflects the use of other nuclear import pathways not typically used during infection, such as those used by the P90A and N74D mutants. Consistent with this hypothesis, we also observed that, similar to the case of reverse transcription inhibition (Extended Data Fig. 8b,c), the N74D and P90A mutants did not induce the relocation of NPC components during infection\(^1\). However, it is unclear why preventing the use of the predominant pathway by wild-type virus would not lead to productive infection in this context. Further characterization of the alternative nuclear import pathway or pathways HIV-1 can use during infection and the development of similar tools to selectively block this pathway, should allow a better understanding of how use of distinct nuclear import pathways drives subsequent nuclear steps of infection of HIV-1.

**Methods**

**Cell lines.** THP1, CEM and SupT1 cells were obtained from ATCC. The 293Ts and HeLa cells used in this study were cultured in DMEM and THP1, CEM and SupT1 cells were cultured in Roswell Park Memorial Institute (RPMI) medium (Cellgro). Both media were supplemented with 10% characterized FCS, 1000 U ml\(^{-1}\) of penicillin, 1000 U ml\(^{-1}\) of streptomycin and 10 µg ml\(^{-1}\) of ciprofloxacin hydrochloride. The cell lines used in this study were monitored for Mycoplasma contamination by utilizing the 4,6-diamidino-2-phenylindole (DAPI) stain. THP1 cells were differentiated by treating the cells with 100 ng ml\(^{-1}\) of phorbol 12-myristate 13-acetate (Sigma-Aldrich) for 48 h. Cells were cultured in normal media without phorbol 12-myristate 13-acetate for another 24 h before performing experiments. To generate MDMs, peripheral blood mononuclear cells were obtained from peripheral blood immediately after collection by layering over lymphocyte separation medium (Corning) and spun at 400g for 15 min. Peripheral blood mononuclear cells were washed twice PBS and cryopreserved in the EasySep Human CD14 Positive Selection Kit (STEMCELL Technologies) according to the manufacturer's protocol. Isolated monocytes were resuspended in RPMI medium supplemented with 10% FCS, 1000 U ml\(^{-1}\) of penicillin, 1000 U ml\(^{-1}\) of streptomycin and 10 µg ml\(^{-1}\) of ciprofloxacin hydrochloride. Primary monocytes were differentiated into macrophages by resuspending in RPMI medium containing 50 ng ml\(^{-1}\) of granulocyte-macrophage colony-stimulating factor (R&D Systems) and 25 ng ml\(^{-1}\) of granulocyte colony-stimulating factor (R&D Systems). MDMs were differentiated for 10 d before the experiments. To isolate primary CD4\(^+\) T cells from peripheral blood, peripheral blood mononuclear cells were isolated as described earlier and CD4\(^+\) cells were isolated using the EasySep Human CD4\(^+\) T Cell Enrichment Kit (STEMCELL Technologies) and were resuspended in RPMI medium as stated earlier. Isolated CD4\(^+\) cells were stimulated with 2.5 µg ml\(^{-1}\) of anti-CD3 antibody (catalogue no. 14-0397-82; Invitrogen), 2.5 µg ml\(^{-1}\) anti-CD28 antibody (catalogue no. 553726; BD Biosciences) and 10 ng ml\(^{-1}\) of interleukin-2 (IL-2; catalogue no. 202-HL; R&D Systems) for 2 d before the experiments. The human blood obtained in this study was from healthy anonymous donors (Loyola University institutional review board no. 208423) and verbal consent was obtained from participants.

**Constructs.** The Nup62DG construct expressing mouse Nup62 was a gift from K. J. Verhey. The mouse Nup62 in the construct was replaced with the open reading frame from hNup62 (plasmid no. 23559; Addgene) and the resulting hNup62DG construct was recloned into the lentiviral backbone pLVX (Clontech). To express the construct in primary CD4\(^+\) T cells and T-cell lines, the hNup62DG construct was cloned into the lentiviral backbone pLVX to mCherry. EnvlGFP reporter virus to mCherry. Δ

**Virus and vector production.** To generate HIV-1 particles pseudotyped with VSV-G, 293T cells seeded in a 15-cm dish at 70% confluency were transfected with 7 µg of pCMV-ΔEnvCherry and NIK was monitored after HD was added (red), as described in Fig. 2. To disrupt the assembled capsids, cells were incubated with 10 µM of PF74 at different time points post-synchronized infection as shown (orange) and was washed off at 24 h. Infectivity was measured as described in Fig. 2. The bar graphs represent data from a single experiment and the line graphs represent normalized and averaged data (± s.e.m.) as described in Fig. 2 from three independent experiments.

![Graphs](image-url)
Generation of stable cell lines and transduction of primary macrophages and CD4+ T cells. To generate the HeLa and THP1 cell lines stably expressing the hNup62DG construct, viral vectors produced using the pLVX-hNup62DG were used for transduction. To select for the stably transduced population, cells were treated with 2 µg ml\(^{-1}\) of puromycin (Gibco) 48 h after transduction. Viral vectors produced from PAd-hNup62DG were used to transduce CEM and SupT1 cells. For these cells, selection was carried out using 1 µg ml\(^{-1}\) puromycin.

To transduce MDMs with the pLVX-hNup62DG, primary monocytes undergoing differentiation to macrophages were treated with simian immunodeficiency virus Vpx virus-like particles on days 5 and 6; then, cells were synchronously infected with a concentrated stock of Nup62 vector.

On day 7, vector-containing medium was replaced with normal medium containing granulocyte-macrophage colony-stimulating factor and macrophage colony-stimulating factor. On day 10 (4 d after transduction), MDMs expressing colony-stimulating factor. On day 10 (4 d after transduction), MDMs expressing hNup62DG construct were synchronously infected with concentrated stock of the HIV-1 strain R7ΔEnvmCherry pseudotyped with VSV glycoproteins, and the NIK assay was performed. Infectivity was measured 96 h after synchronized infection.

To transduce CD4+ T cells, cells were stimulated for 2 d with anti-CD3/CD28 and IL-2; then, they were transduced with a concentrated stock of PAd-hNup62DG and spinoculated at 800 g for 1 h at 32 °C. Vector-containing media was changed 24 h later. Four days after transduction, CD4+ T cells expressing the hNup62DG construct were synchronously infected with concentrated stock of the HIV-1 strain R7ΔEnvmCherry pseudotyped with VSV glycoprotein and the NIK assay was performed as described in the next section. Infectivity was measured 48 h post-synchronized infection.

HIV-1 NIK assay. The NIK assay was performed as outlined in Fig. 2a. Cells stably transduced or transduced with the hNup62DG construct were plated in 48-well plates. Dividing cells were pretreated overnight with 10 µg ml\(^{-1}\) of aphidicolin to stop cell division and ensure viral entry through the NPC. Cells were synchronously infected with the HIV-1 strain R7ΔEnvmCherry pseudotyped with VSV-G or HIV-1 envelope glycoproteins by spinoculation at 13 °C for 2 h at 1,200 g. After spinoculation, medium was replaced with warmed 37 °C normal medium. HD drug at a final concentration of 1.5 µM was added to block active nuclear pore transport immediately after synchronized infection (time point 0 h) or added at the indicated time post-infection as shown in Fig. 2a. Twenty-four hours post-synchronized infection, HD-containing medium was removed, cells were washed and cultured in normal medium. Infectivity was measured 48 h post-synchronized infection for all cells except MDMs, where infectivity was measured 96 h post-infection. Infectivity was determined by measuring the percentage of mCherry+ cells using the BD LSRFortessa flow cytometer (BD Biosciences). The percentage of mCherry+ cells was calculated as the ratio of the double-positive population (Nup62DG+, R7mCherry+) over the total amount of Nup62DG+ population (Extended Data Fig. 2b).

Antibodies and chemicals. The HIV-1 capsid protein, p24, was stained using either anti-HIV p24 monoclonal reagent (183-H12-5C; HIV-1 p24 hydrdroma from B. Chesebro) and obtained through the National Institutes of Health (NIH) AIDS Reagent Program, Division of AIDS, NIAID, NIH or using HIV-1 p24 antibody (24-4) from Santa Cruz Biotechnology (catalogue no. sc-9728). The rabbit polyclonal antibodies against lamin A/C (catalogue no. 10398-1-AP) and Nup62 (catalogue no. 13916-1-AP) used for the immunofluorescence experiments were purchased from Proteintech. The rabbit polyclonal antibody against Nup358 (catalogue no. ab64276) was purchased from Abcam. The mouse monoclonal antibodies against Nup62 (catalogue no. sc-68389) and GAPDH (catalogue no. sc-25778) were purchased from Santa Cruz Biotechnology. Secondary antibodies conjugated to a fluorophore were used for the immunofluorescence studies and were purchased from Jackson Immunoresearch. The B/B HD drug was purchased from Clontech (catalogue no. 635059). PEF4, NVP, E2 and the DAPI stain were obtained from Sigma-Aldrich. Aphidicolin was purchased from Cayman Chemical.

Western blotting. Cell lysates were prepared by lysing cells with NP-40 lysis buffer (100 mM of Tris pH 8.0, 1% NP-40, 150 mM of NaCl) containing protease inhibitor cocktail (Roche) for 10 min on ice. Following incubation, lysates were spun down at 13,000 rpm for 10 min and supernatant was collected for western blot analysis. In both Nup62 and Nup358, cells were purchased from Santa Cruz Biotechnology. Secondary antibodies conjugated to a fluorophore were used for the immunofluorescence studies and were purchased from Jackson Immunoresearch. The B/B HD drug was purchased from Clontech (catalogue no. 635059). PEF4, NVP, E2 and the DAPI stain were obtained from Sigma-Aldrich. Aphidicolin was purchased from Cayman Chemical.

Microscopy and image acquisition. Z-stack images were collected with a DeltaVision widefield fluorescence microscope (Applied Precision) equipped with a digital camera (CoolSNAP HQ; Photometrics) and a 1.4-numerical aperture 100× objective lens. Excitation light was generated with an Insight SSI solid-state illumination module (Applied Precision); images were deconvolved with the SoftWoRx deconvolution software v7.0.0 (Applied Precision, GE Healthcare). In all experiments, identical conditions were used to acquire all images. After deconvolution, images were analysed using Imaris v.8.4.1 (Bitplane). An algorithm was used to count and using the surface area of all pixels to estimate the signal of interest; the maximum fluorescence intensity detected within these surfaces was measured. The same algorithm was applied to all images within an experiment. For the live cell experiments, cells were plated on delta DPG dishes (Thermo Fisher Scientific) and maintained at 5% CO2 and 37 °C in an environmental chamber on a DeltaVision microscope.

Quantitative PCR with reverse transcription (RT–qPCR). Cells were infected with equal multiplicity of infection and RT–qPCR was performed to determine the relative expression of the late reverse transcription and 2-LTR products. The following primers targeting the HIV-1 DNA polymerase were used for the late reverse transcription: polymerase forward, 5′-GGGAGCCACACAAATGAA-3′ and polymerase reverse, 5′-CCAGGGCTCTAGTCGGATC-3′. The 2-LTR primers used were described previously30. GAPDH was used as the housekeeping gene for normalization. In brief, genomic DNA from cells was extracted according to the DNeasy Blood and Tissue Kit protocol (QIAGEN). DNA concentration was determined and equal amounted was digested with DpnI (New England Biolabs) before performing RT–qPCR.

In situ viral DNA (vDNA) detection. To detect negative and positive HIV-1 vDNA strands, probes targeting the antisense (detect negative vDNA) or sense strand (detect positive vDNA) and the detection reagents were purchased from Advanced Cell Diagnostics. Negative vDNA was detected using the Advanced Cell Diagnostics probe HIV-gagpold-sense (catalogue no. 317701) and positive vDNA was detected using the Advanced Cell Diagnostics probe HIV-C2 (catalogue no. 446211-C2). The assay was performed according to the manufacturer's protocol and as described previously31. Briefly, following fixation with 3.7% paraformaldehyde, cells on coverslips were dehydrated with the sequential addition of 50, 70 and 100% ethanol for 5 min each at room temperature. To rehydrate cells, the sequential addition of ethanol was reversed and cells were incubated with each solution for 2 min at room temperature. Following rehydration, cells were treated with protease solution (Pretreat 3) diluted in PBS (1:2 dilution) for 15 min in a humified HyBE EZ oven (Advanced Cell Diagnostics) at 40 °C. After protease treatment, samples were washed three times with molecular grade water and then treated with 5 mg ml\(^{-1}\) of RNase A (QIAGEN) in PBS for 30 min at 37 °C. Cells were then washed three times with molecular grade water and heated at 55 °C for 30 min with hybridization buffer as described by Puray-Chavez et al. The hybridization buffer was removed and probes were diluted in hybridization buffer (1:1 for negative vDNA C1 probe and 1:50 for positive vDNA C2 probe) and allowed to hybridize with samples in the humified HyBE EZ oven at 40 °C for 2 h. Following hybridization with the samples, the probes were visualized by hybridizing with preamplifiers, amplifiers and a fluorescent label as outlined in the manufacturer’s protocol for probe detection (RNAseq Multiplex Fluorescent Detection Kit; Advanced Cell Diagnostics).

Proximity ligation assay. The Duolink PLA kit was purchased from Sigma-Aldrich and the assay was performed as described previously32. Briefly, cells were grown on coverslips and fixed with 3.7% paraformaldehyde. To detect the interaction between viral capsid protein, p24 and Nup358, cells were processed for immunofluorescence staining and incubated in 5% BSA followed by incubation with primary antibodies targeting viral protein p24 (mouse monoclonal) and Nup358 (rabbit polyclonal). After primary staining, coverslips containing cells were washed and incubated (1 h at 37 °C) with secondary anti-mouse conjugated with minus and anti-rabbit conjugated with plus Duolink II PLA probes. Coverslips were washed again and incubated with ligation/digase solution (30 min at 37 °C) followed by washing and subsequent incubation with amplification-polymerase solution (100 min at 37 °C) containing Duolink In Situ Detection Reagents Red. Finally, coverslips were washed and mounted with Duolink II mounting medium containing DAPI. Interactions were detected as fluorescent spots (λ(excitation/emission) = 598/634 nm) with a fluorescence microscope.

Quantification of perinuclear and cytoplasmic protein signals. Quantification of p24, Nup358 or Nup62 signals in the cell was performed following a similar quantification method described in our previous study33. The nuclear and perinuclear signals were determined using surface masks around the nucleus based on

of packaging plasmid pSPAX and 5 µg of pCMV-YS-G. Viruses were collected 48 and 72 h post-transfection and filtered through a 0.45-µm filter (Merck Millipore). Viruses and viral vectors were concentrated by spinning at 4 °C overnight at 5,000 g. Synchronized infection of viruses and viral vectors on cells was performed by spinoculation at 13 °C for 2 h at 1,200 g.
on the DAPI channel. To detect the perinuclear and nuclear signals, an algorithm was created that reliably overestimates the size of the nucleus, as described previously (Extended Data Fig. 3a). The perinuclear algorithm to detect cell nuclei included all z-sections acquired. All events within this mask were considered cytoplasmic, as determined using the surface function and masking tool in Imaris v. 8.4.1.

To detect the nuclear signal, an algorithm was designed to detect the cell nuclei using the DAPI channel as reference and the surface function in Imaris v. 8.4.1 (Extended Data Fig. 3b). Z-sections close to the top or bottom of the nucleus were excluded to avoid detection of extranuclear signals. All events within the mask were considered nuclear and all events outside the mask were considered perinuclear and cytoplasmic. The amount of perinuclear signal was quantified by subtracting the signal obtained from the nuclear and perinuclear signals to the signal obtained from the nucleus.

Statistical analysis. Prism 6.00 (GraphPad Software) was employed for statistical analysis and to create the graphs. Statistical significance was assessed using a two-way analysis of variance (ANOVA) and Bonferroni post-test. P < 0.05 was considered significant. Data were represented as the mean ± S.E.M. depending on the graph. All statistics for the NIK assays are provided as source data.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The Nup62-DmrB-GFP plasmids used in this study have been deposited with Addgene; relevant information is provided in the Methods. All data generated or analysed during this study are presented in the paper, in the supplementary information or as source data. All data are available from the corresponding author upon reasonable request. Correspondence and requests for materials should be addressed to E.M.C. Source data for Figs. 3 and 4 and Extended Data Figs. 5 and 8 are included with this paper.

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Author contributions

A.D. and E.M.C. designed the experiments. A.D., N.B., S.T. and V.Z. conducted the experiments. A.D., N.B., S.T. and E.M.C. analysed the data. A.D. and E.M.C. wrote the manuscript. E.M.C. supervised the study.

Competing interests

The authors declare no competing interests.

Additional information

Extended data is available for this paper at https://doi.org/10.1038/s41564-020-0735-8.

Supplementary information is available for this paper at https://doi.org/10.1038/s41564-020-0735-8.

Correspondence and requests for materials should be addressed to E.M.C.

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Extended Data Fig. 1 | Expression of the Nup62 dimerization construct in cells does not alter normal cell physiology. **a**, HeLa cells were transduced simultaneously with lentiviral vectors driving expression of Nup62DG or mCherry-estrogen receptor alpha (ERα). Imaging fields were selected that contain cells expressing only ERα (marked by asterisk) or both ERα and Nup62DG. Live cell imaging was performed after addition of estradiol (E2) to mediate nuclear translocation of ERα. Images acquired every four minutes for a total of one hour. **b**, Similar experiment as in (**a**) except the cells were treated with the homodimerizing drug (HD) to block NPC transport along with E2 treatment. **c**, Accumulation of mCherry-ERα fluorescence in the nuclear region was monitored in Nup62DG+ and Nup62DG- cells treated with E2 using ImageJ plugin Time Series Analyzer V3. Depicted mean values from three independent experiments (±SEM). **d**, similar quantification as in (**c**) in cells treated with E2 and HD. Error bar represents SEM. Statistical significance was assessed using Two-way ANOVA and Bonferroni post test. P<0.05 was considered significant in our experiments.
**Extended Data Fig. 2** | Quantification strategy employed to quantify nuclear, perinuclear and cytoplasmic signal using Imaris. Schematic representation of the quantification technique used in this manuscript with an example image of a cell expressing the Nup62DG and infected with HIV-1. Cells were stained for the capsid protein p24 and DAPI. 

**a.** Nuclear and perinuclear signal was quantified using a DAPI mask generated using the surface function in Imaris that exceeded the boundary of DAPI stain to include the perinuclear signal. As depicted, all signal within this mask was considered nuclear and perinuclear signal. Similarly, all signal outside of this mask was considered cytoplasmic signal.

**b.** To focus on exclusively nuclear signal, an algorithm which reliably identified the nuclear boundary, as indicated by a DAPI stain, was generated in Imaris. Sections close to the upper and lower boundary of the nucleus (in Z) were removed to focus analysis on nuclear events. As in (a) signal within this mask was considered nuclear and all signal outside this mask was considered cytoplasmic and perinuclear.
Extended Data Fig. 3 | HIV-1 infection induces Nup62 relocalization and colocalization with HIV-1 cores in the cytoplasm. 

a, Nup62 localization in uninfected HeLa cells. 
b, HeLa cells synchronously infected with VSVG: R7ΔEnv:Cherry at 3 h post infection, fixed and stained for Nup62 (green), HIV-1 capsid protein p24 (red), and DAPI (blue) for cell nuclei. Enlarged view of colocalization (boxed region) between Nup62 and HIV-1 capsid protein p24 shown in the bottom panel and indicated by arrows. 
c, Quantification of cytoplasmic Nup62, as described in methods section and Supplementary Fig. 2a. 20 or more cells analyzed per sample. Data averaged from three independent experiments. Error bar represents SEM. 
d, Quantification of the percent p24 colocalizing with Nup62 in HeLa cells. 20 or more cells analyzed per sample. Data averaged from three independent experiments. Error bar represents SEM. Statistical significance was assessed using Two-way ANOVA and Bonferroni post test. P<0.05 was considered significant in our experiments.
Extended Data Fig. 4 | HIV-1 particles retained at the NPC over extended period upon Nup62 dimerization. HeLa cells expressing Nup62DG synchronously infected with Gag-Integrase-Ruby (GIR) labeled HIV-1 particles. 1 hour following synchronized infection, cells were treated with homodimerizing drug (HD) and imaged every 4 minutes for 1 hour. Snapshot of a GIR labeled virus particle (boxed region) at the indicated times during acquisition is shown. Similar pattern observed across 5 independent experiments.
Extended Data Fig. 5 | Monitoring reverse transcription with a low dose of RT inhibitor nevirapine to mirror the level of inhibition induced by Nup62DG blockade. THP1 cells expressing the Nup62DG were differentiated to macrophages and infected with VSVg-ΔEnvmCherry and NIK monitored by HD addition (red) as described in Fig. 2. To monitor replication kinetics, cells incubated with HIV-1 reverse transcriptase inhibitor Nevirapine (NVP, 1.6 µM) at different time’s post infection as depicted (green) and washed off at 24 h. Infection measured as described in Fig. 2. Bar graphs depict data from a single experiment and line graphs depict normalized and average data (±SEM) as described in Fig. 2 from three independent experiments. NT represents no treatment.
Extended Data Fig. 6 | HIV-1 reverse transcription completes in the nucleus of infected T cells. a-b, CEM and SupT1 expressing the Nup62DG infected with VSVg-R7ΔEnvmCherry and NIK monitored by HD addition (red) as described in Fig. 2. To monitor replication kinetics, cells incubated with HIV-1 reverse transcriptase inhibitor Nevirapine (NVP, 5µM) at different time’s post infection as depicted (green) and washed off at 24 h. Infection measured as described in Fig. 2. Similar experiment as above done on SupT1 cells expressing Nup62DG after infection with R7ΔEnvmCherry pseudotyped with HIV-1 envelope from the HXB2 strain. All bar graphs depict data from a single experiment and line graphs depict normalized and average data (±SEM) as described in Fig. 2 from three independent experiments. NT represents no treatment.
Extended Data Fig. 7 | Positive strand HIV-1 vDNA colocalize with Negative strand only in the nucleus of infected THP1 differentiated macrophages and primary macrophages. a, b, THP1 differentiated macrophages (a) and monocyte derived macrophages (b) synchronously infected with VSVg-R7ΔEnv-mCherry and fixed at different times post synchronized infection. Cells treated with RNase A and stained for (-) VDNA (orange) and (+) vDNA (red) using specific sense and antisense probes. Upon probe staining, these cells were also stained for HIV-1 capsid protein p24 (green) and nuclear Lamin A/C (blue). Depicted a representative image at the indicated time points. Data shown here is representative of three independent experiments. Quantification provided in Fig. 3d.
Extended Data Fig. 8 | Nup358 relocalization induced upon HIV-1 infection absent upon inhibition of HIV-1 reverse transcription.  

**a**, HeLa cells expressing the Nup62DG infected with VSVg- R7ΔEnvmCherry and NIK monitored by HD addition alone (red) or in the presence of NVP treatment for the first 6 hours (blue) following synchronized infection. Bar graphs depict data from a single experiment and line graphs depict normalized and average data (± SEM) as described in Fig. 2 from three independent experiments. 

**b**, HeLa cells and primary human macrophages (MDM) synchronously infected with HIV-1 (WT) and treated with HIV-1 reverse transcriptase inhibitor NVP. Cells fixed 0, 1 and 3 h (shown) post infection and stained for Nup358 (green), HIV-1 capsid protein p24 (red), and DAPI (blue). Middle panel depicts colocalization between Nup358 and p24. Data shown here is representative of three independent experiments. 

**c,d**, Percent Nup358 in the cytoplasm and percent p24 colocalizing with Nup358 at the different times post infection in HeLa and MDM. 20 or more cells analyzed in each experiment. Data averaged (mean) from three independent experiment. 

**e**, Proximity ligation assay performed in HeLa and MDM after HIV-1 infection and cells fixed 3h post infection. Each red puncta represents a positive PLA signal generated by interaction of Nup358 and p24. 

**f**, Average fold increase in PLA signal, relative to uninfected control, from three independent experiments. 20 or more cells analyzed in each experiment. Error bar represents SEM. All statistical significance was assessed using Two-way ANOVA and Bonferroni post test. P<0.05 was considered significant in our experiments.
Extended Data Fig. 9 | Nuclear p24 signal upon inhibition of reverse transcription. a, HeLa cells expressing Nup62DG infected with VSVg-ΔEnvmCherry and treated with HIV-1 reverse transcriptase inhibitor NVP for 7 hours following synchronized infection. Cells fixed and stained for HIV-1 capsid protein p24 (red), and DAPI (blue). b, Quantification of nuclear HIV-1 p24 signal, performed as described in methods and Supplementary Fig. 2b. 20 or more cells analyzed in each experiment. Data averaged from three independent experiments. Error bar represents SEM. Statistical significance was assessed using Two-way ANOVA and Bonferroni post test. P<0.05 was considered significant in our experiments.
Extended Data Fig. 10 | Insensitivity of the capsid mutants N74D and P90A to the Nup62 mediated artificial nuclear pore block suggests heterogeneity in the nuclear pores and usage by WT virus. HeLa cells stably expressing the Nup62DG infected with HIV-1 harboring either the WT capsid (blue) or capsid mutants N74D (red) and P90A (orange). NIK measured in these cells by HD addition as described in Fig. 2. a, Shown a bar graph from a single experiment. b, Percent inhibition attained upon blocking the nuclear pore for the first 24 h. Data averaged from three independent experiments. Error bar represents SEM.
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- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
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- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

Images acquisition and image deconvolution were performed by Softworx 7 program from DeltaVison. Image analysis was performed with imaging software Imaris 8.4.1 from Bitplane and ImageJ from NIH. Western blot images were processed using ImageJ from NIH.

Data analysis

Image data analysis are described in the methods section and performed with imaging software Imaris 8.4.1. Algorithm to analyze images were designed using the surface function feature in Imaris. The algorithm was applied to every images from an experiment using the Batch function in Imaris. Graphs were generated using PRISM 6 from GraphPad. Statics used for all experiments were performed using PRISM 6.

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The Nup62-DmrB-GFP plasmids used in this study are deposited to Addgene and relevant information provided in the methods section. All data generated or analyzed during this study are presented in the paper or in the supplementary information. All data are available from the corresponding author upon reasonable request.
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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size
All experiments in this study were at least done in triplicates with reproducible data and in some cases more than three experiments were done. Sample sizes were determined based on the Authors experience to generate a convincing and compelling result.

Data exclusions
Experiments where the longer incubations (0-24h) of HD treatment did not give sufficient inhibition of infection (75-80% inhibition) was excluded. This normally happened when the cell lines used were in culture for a long time (>4weeks). Fresh cells were then thawed for subsequent experiments.

Replication
The findings in this paper were highly reproducible. When the cell lines used are in a healthy state and when the longer HD inhibition as outlined above worked, the results were consistent across all experiments.

Randomization
N/A

Blinding
None of the Flow data was blinded as data were quantitative and measurements made using a machine. However microscopic experiments shown in Extended Data Fig 3 and Extended data Fig 7 were blinded by a person other than the experimenter to provide an more unbiased analysis.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

- n/a
- Involved in the study

- Antibodies
- Eukaryotic cell lines
- Palaeontology
- Animals and other organisms
- Human research participants
- Clinical data

Methods

- n/a
- Involved in the study

- ChIP-seq
- Flow cytometry
- MRI-based neuroimaging

Antibodies

| Antibodies          | Supplier                      | Catalog Number |
|---------------------|-------------------------------|----------------|
| 183-H12-5C         | NIH AIDS Reagent Program      | 3537           |
| HIV-1 p24 (24-4)   | Santa Cruz Biotechnology      | sc-69728       |
| Lamin A/C          | Proteintech                   | 10298-1-AP     |
| Nup52              | Proteintech                   | 13916-1-AP     |
| Nup358             | Abcam                         | ab64276        |
| Nup62              | Santa Cruz Biotechnology      | sc-48389       |
| GAPDH              | Santa Cruz Biotechnology      | sc-47724       |
| Human CD3          | Invitrogen                    | 14-0037-82     |
| Huanm CD28         | BD Biosciences                | 555726         |

Validation

Most of the antibodies were validated from the manufacture and the required validation including western blots, immunofluorescence images are provided in the manufacture website. HIV-1 p24 antibodies were validated in our laboratory using Western blots and immunofluorescence on human cells and infected with HIV-1. The p24 antibody 183-H12-5C has also been cited in our labs previous publication.
Eukaryotic cell lines

Policy information about cell lines

| Cell line source(s) | HeLa, 293Ts, THP1, CEM and SupT1 cell lines used in this study are from ATCC |
|---------------------|-----------------------------------------------------------------------------|
| Authentication      | Microscopic inspection                                                      |
| Mycoplasma contamination | Cell lines stained for DAPI to look for mycoplasma contamination (visible by DAPI staining in the cytoplasm). No contamination observed in the cell lines used. |
| Commonly misidentified lines (See ICLAC register) | N/A |

Flow Cytometry

Plots

Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

| Sample preparation | Samples harvested and resuspended in PBS containing 1% paraformaldehyde |
|-------------------|-------------------------------------------------------------------------|
| Instrument         | BD LSRRFortessa cytometer from BD Bioscience                           |
| Software           | Flow cytometry data were analyzed using FlowJo V10 and using online free software Flowing software (http://flowingsoftware.btk.fi/) |
| Cell population abundance | Flow cytometer was set to acquire at least 10,000 events. In case of CD4+T cell experiments, the number of events was increased to 50,000 events. |
| Gating strategy    | For all Flow data, from the FSC/SSC plots, live cells were gated and from these live cell population, single cells were gated based on FSC-A/FSC-H. Now from these live/single cell population, dots plots were generated with X axis representing GFP (Nup62 construct) and Y axis representing mCherry (HIV-1 infection). Example for the gating strategy provided in Supplementary Figure 1. |

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.