Supplementary Figures and Legends

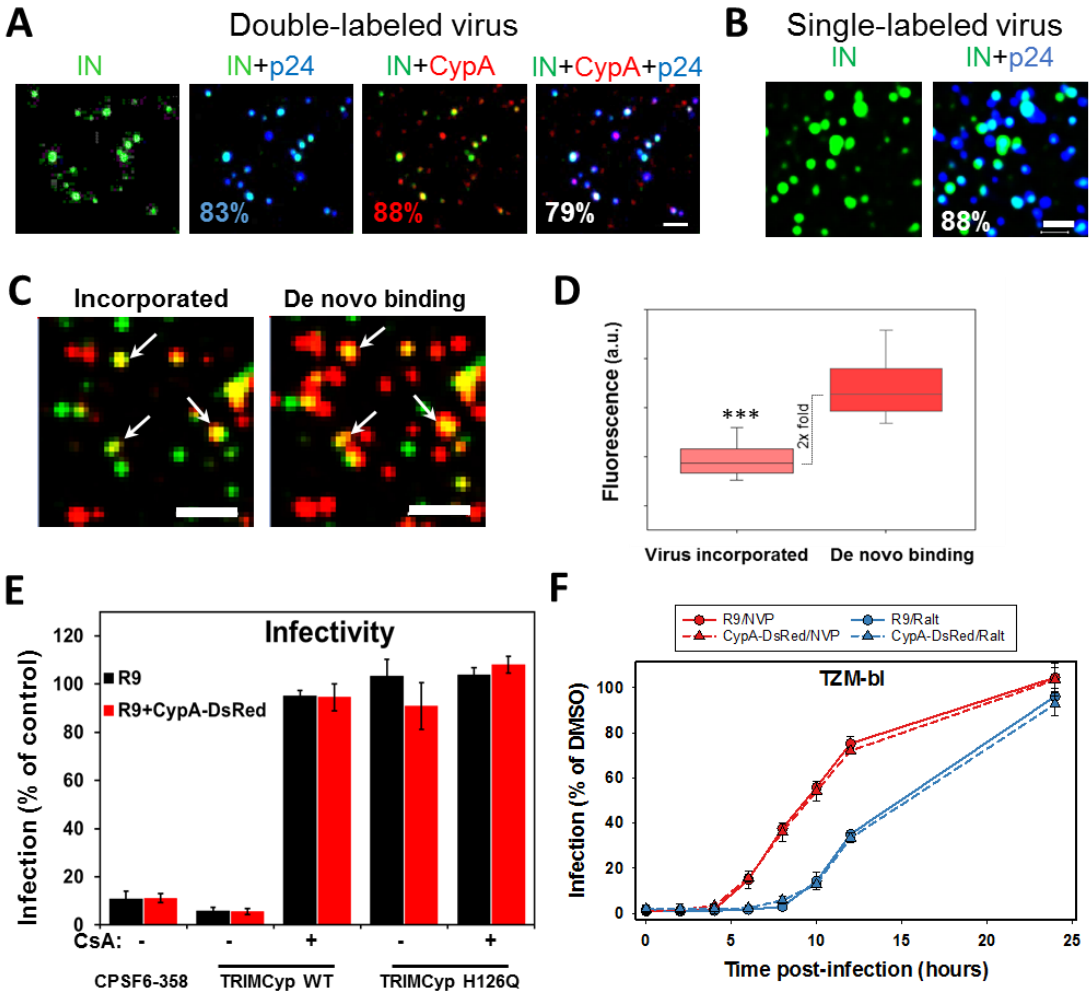

Figure S1. Related to Figures. 1 and 2. Validation of CypA-DsRed as a marker for HIV-1 uncoating. (**A** and **B**) VSV-pseudotyped HIV-1 particles labeled with INsfGFP (single-labeled) or co-labeled with INsfGFP/CypA-DsRed (double-labeled) were bound to poly-L-lysine treated cover glass for 30 min at 4^{o}C, fixed, immunostained for p24 and imaged. The fraction of total INsfGFP puncta co-localized with CA/p24 and with CypA-DsRed are shown in blue and red, respectively. The fraction of CA/p24-CypA-DsRed that colocalized with total INsfGFP puncta is shown in (A) white label. (**C**) CypA-DsRed is incorporated into HIV-1 at sub-stoichiometric levels. INsfGFP/CypA-DsRed labeled HIV-1 was immobilized on cover glass, as described above, permeabilized with saponin (100 µg/ml) for 30 sec and imaged. Lysate from 293T cells transfected with CypA-DsRed was collected from digitonin-permeabilized cells and added (estimated concentration of CypA-DsRed 0.5 µM) to viruses. *De novo* CypA-DsRed binding to permeabilized cores was visualized by time-lapse imaging for 5 min. Images of intact viruses with incorporated CypA-DsRed and permeabilized particles allowed to bind exogenous CypA-DsRed are shown. Arrows indicate cores that show increase in CypA-DsRed signal above the original level. (**D**) The fluorescence intensity of viral CypA-DsRed prior to cytosol addition and 5 min after (n≥1,200 cores). (**E**) CypA-DsRed incorporation does not perturb CA/host-factor interaction in target cells. TZM-bl cells were engineered to express the CPSF6-358 cytosolic fragment or the TRIMCyp restriction factors that bind non-overlapping sites at the CA/hexamer interface. Cells were inoculated with VSV-pseudotyped HIV-1 particles that were unlabeled or

labeled with CypA-DsRed in the presence or absence of 2 μM CsA (as noted). At 48 h.p.i., infectivity was measured by a luciferase assay. The results show equal inhibition of unlabeled and CypA-DsRed labeled viruses in the absence of CsA. (**F**) CypA-DsRed incorporation into HIV-1 does not affect the kinetics of HIV-1 entry. HIV-1 pseudoviruses unlabeled or labeled with CypA-DsRed were bound to TZM-bl cells (MOI 0.2) by spinoculation (1200xg for 30min at 4^{o}C) and excess virus was washed off. Infection was initiated by shifting to 37^{o}C and the drugs, nevirapine (10 μM) to block reverse transcription, or Raltegravir (10 μM) to block integration, or DMSO (control) were added at indicated time points post-infection. Infection was measured at 48 h.p.i. Error bars in (E) and (F) are SEM from 3 independent experiments. Scale bars 2 μm (A) and (B), are and 5 μm (C).

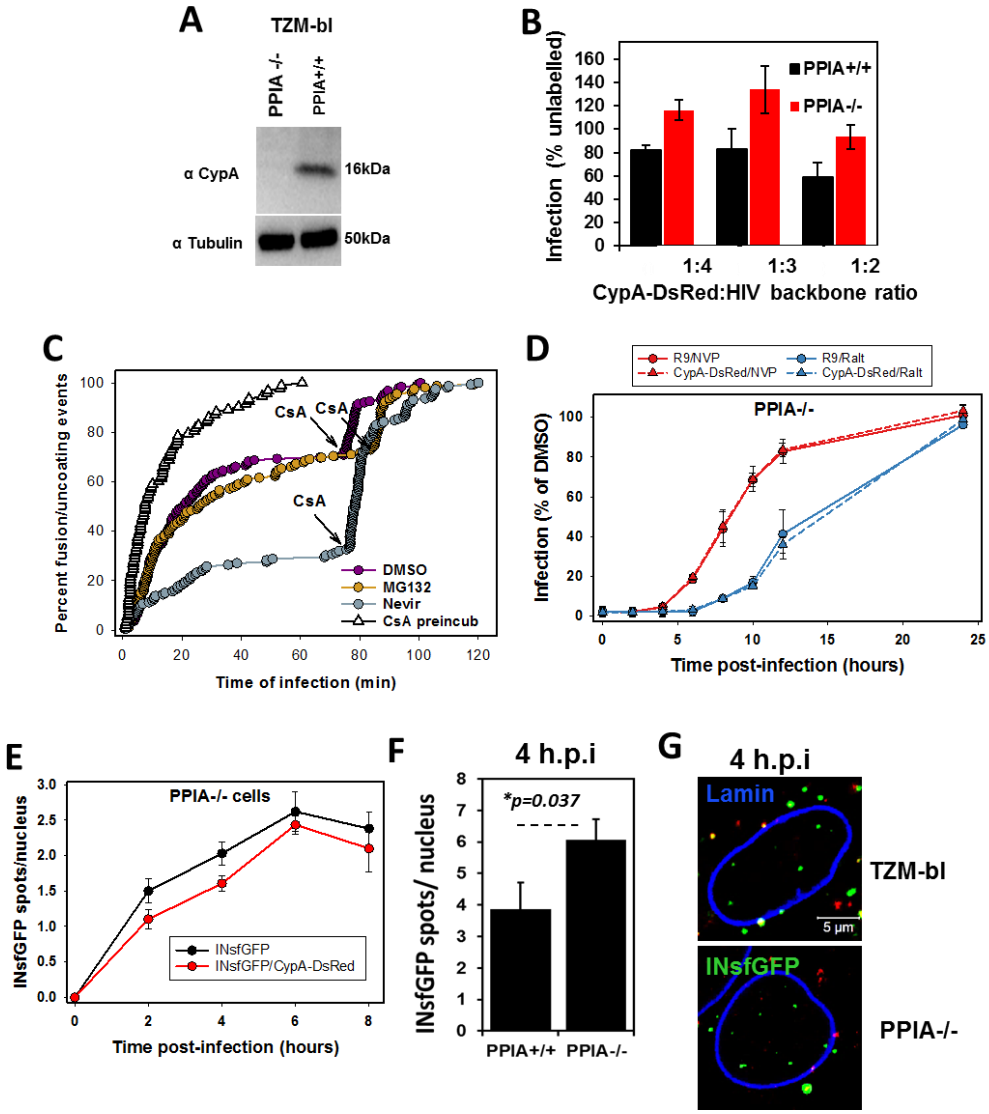

Figure S2. Related to Figures. 1 and 2. Assessment of the role of target cell CypA in HIV-1 infection. (**A**) TZM-bl or PPIA-/- cell lysates were probed for the expression of CypA by Western blotting using anti-CypA antibody. (**B**) CypA-DsRed incorporation into HIV-1 does not affect infectivity of VSV-pseudotyped pR9ΔEnv pseudoviruses produced by co-transfection of different dilutions of the CypA-DsRed plasmid into 293T cells. Infection of TZM-bl and PPIA-/- cells was measured at 48 h.p.i. Percent infection with respect to unlabeled viruses is shown. Error bars are SEM from >5 independent experiments. (**C**) Endogenous CypA modulates early HIV-1 uncoating. INsfGFP/CypA-DsRed labeled VSV-pseudotyped viruses were bound to PPIA-/- cells by spinoculation in the cold (4^{o}C; 1200xg) (MOI 0.008). Infection was synchronized by shifting to 37^{o}C and imaging cells on a temperature-controlled microscope stage at 5% CO_2. The kinetics of single HIV-1 uncoating was determined based upon an abrupt loss of the CypA-DsRed signal, as described in Star Methods. Nevirapine (10 μM) or DMSO were added at 0 min, immediately before image acquisition. Alternatively, cells were pretreated with MG132 (10 μM) for 30 min and imaged in the presence of the drug. CsA (10 μM) was added at indicated time point (arrows). The data are from >5 independent experiments. (**D**) CypA-DsRed incorporation into HIV-1 does not affect kinetics of HIV-1 entry. HIV-1 pseudoviruses unlabeled

or labeled with CypA-DsRed were bound to PPIA-/- cells (MOI 0.2), and experiment was performed as in Suppl.Fig. 1F. (**E**) Kinetics of nuclear import is not altered by CypA-DsRed incorporation into HIV-1. PPIA-/- cells were infected with viral supernatants with equal amounts of RT activity, and infection was stopped at indicated time points by fixation with 2% PFA. Cells were immunostained for Lamin-B1, and the number of nuclear INsfGFP spots was quantified, as described in Methods. Error bars are SEM from 4 fields of view. (**F**) The number of nuclear INsfGFP spots resulting from INsfGFP/CypA-DsRed virus infection of TZM-bl or PPIA-/- cells. A representative experiment from >4 independent experiments is shown. Error bars are SEM from 4 fields of view. (**G**) An example of nuclear INsfGFP complexes in TZM-bl and PPIA-/- cells. Scale bar 5 μm.

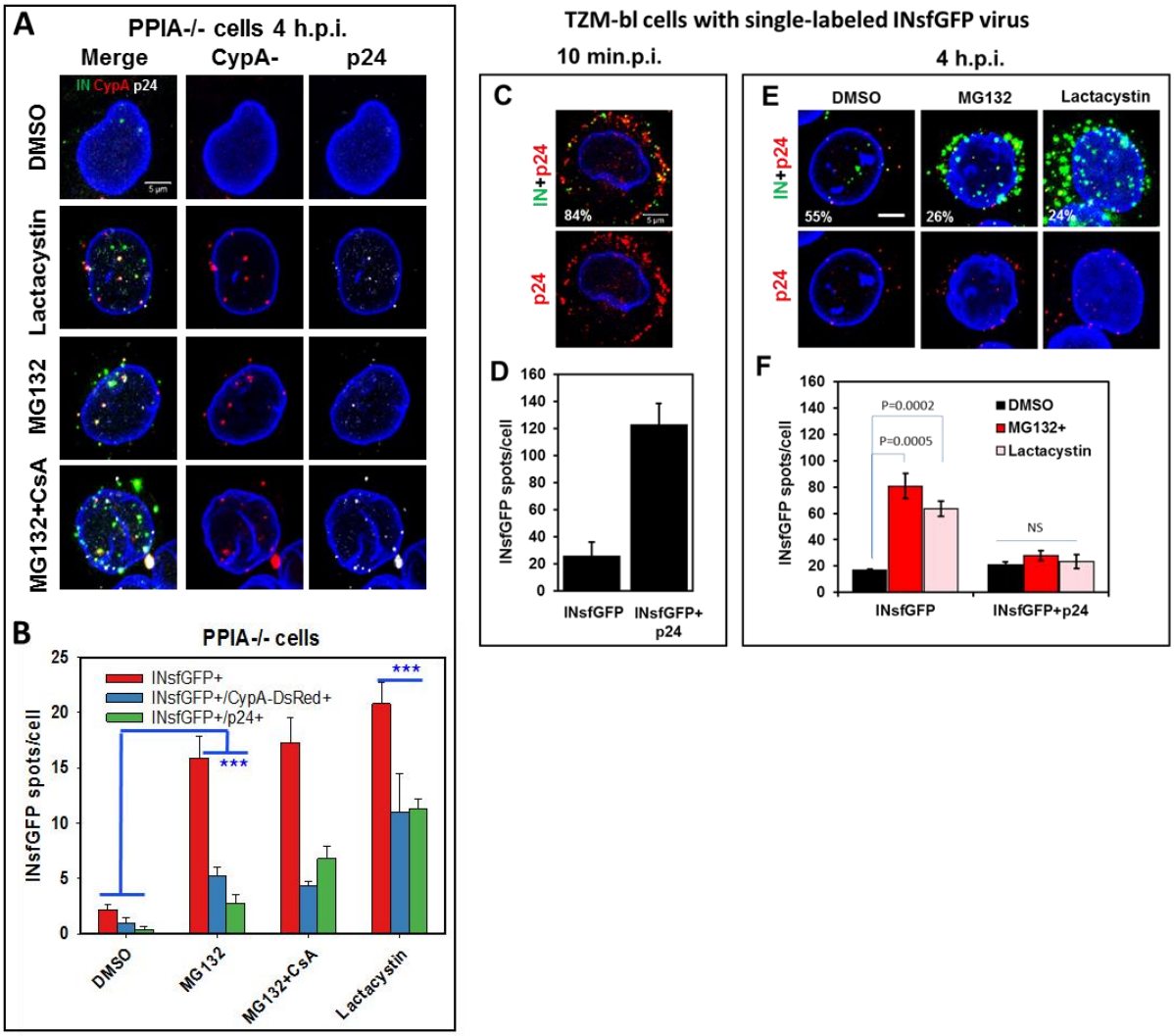

Figure S3. Related to Figure 1. HIV-1 CA protects RTC/PICs from cytoplasmic proteasomal degradation. Cells were pretreated with 10 µM MG132, 10 µM Lactacystin or left untreated (DMSO) and infected with VSV-pseudotyped INsfGFP/CypA-DsRed labeled HIV-1. (**A**) Images of fluorescent HIV-1 complexes in PPIA-/- cells treated with DMSO, MG132 or Lactacystin at 4 h.p.i and immunostained for p24. (**B**) The number of INsfGFP complexes positive and negative for CypA-DsRed or CA/p24 at 4 h.p.i. was determined by analyzing 70 PPIA-/- cells in 2 independent experiments. A representative experiment is shown. Error bars are SEM from 4 fields of view. Post-fusion cores were identified by addition of CsA (10 µM) at the end of the experiment, 30 min prior to fixation of cells. The INsfGFP/CypA-DsRed complexes remaining after CsA treatment correspond to viruses trapped in endosomes. (**C-F**) TZM-bl cells were infected with single labeled (INsfGFP) pseudoviruses for 10 min (C, D) or 4 h (E, F) in the presence of DMSO, MG132 or Lactacystin, as above. Cells were fixed, immunostained and the number of INsfGFP only or colocalized INsfGFP/p24 complexes was quantified from 65 cells in 2 independent experiments. Error bars are SEM from 4 fields of view. Scale bars in C and E are 5 µm.

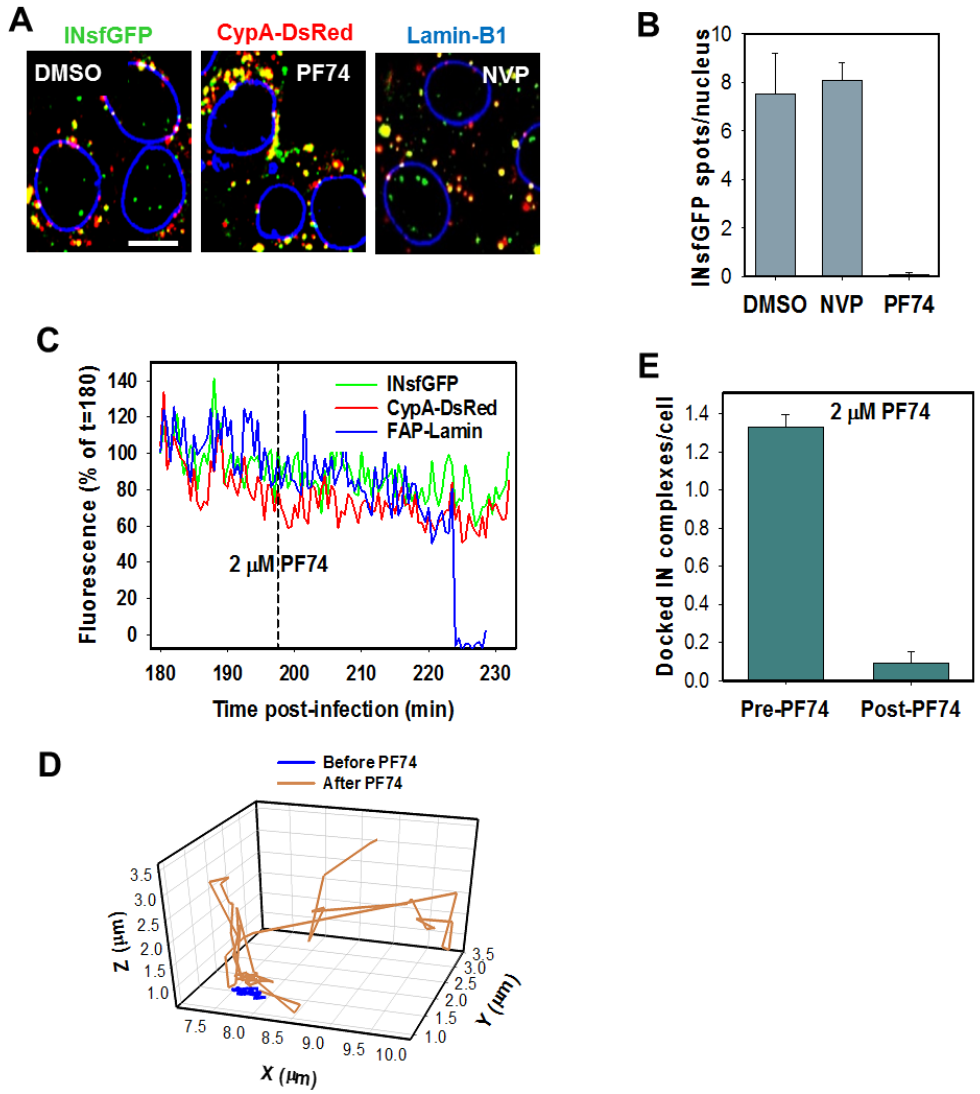

Figure S4. Related to Figure 3. Nuclear import of INsfGFP complexes is independent of reverse transcription and is blocked by PF74 that displaces docked cores. PPIA-/- cells were infected with HIV-1/VSV-G pseudoviruses encoding for eGFP and labeled with INsfGFP/CypA-DsRed (MOI 2) for 6 h in the presence of PF74 (2 µM), nevirapine (NVP, 10 µM) or DMSO (control). (**A**) Images of the cytoplasmic and nuclear particles. Scale bar 10µm. (**B**) Analysis of the experiments in panel A for ~70 nuclei. Error bars are SEM from four fields of view containing >70 nuclei. (**C-E**) INsfGFP/CypA-DsRed labeled VSV-pseudotyped HIV-1 was used to infect FAP-Lamin expressing TZM-bl cells and cells were imaged at 3-4 h.p.i. (C, D) Fluorescence intensity traces for a docked single HIV-1 particle that separates from the Lamin signal after application of PF-74 (2 µM, dotted line) and 3D trajectory of the particle in before and after PF-74 addition. (E) Quantification of docked cores before and after PF-74 (2 µM) treatment. Error bars in (E) are SEM from 4 fields of view.

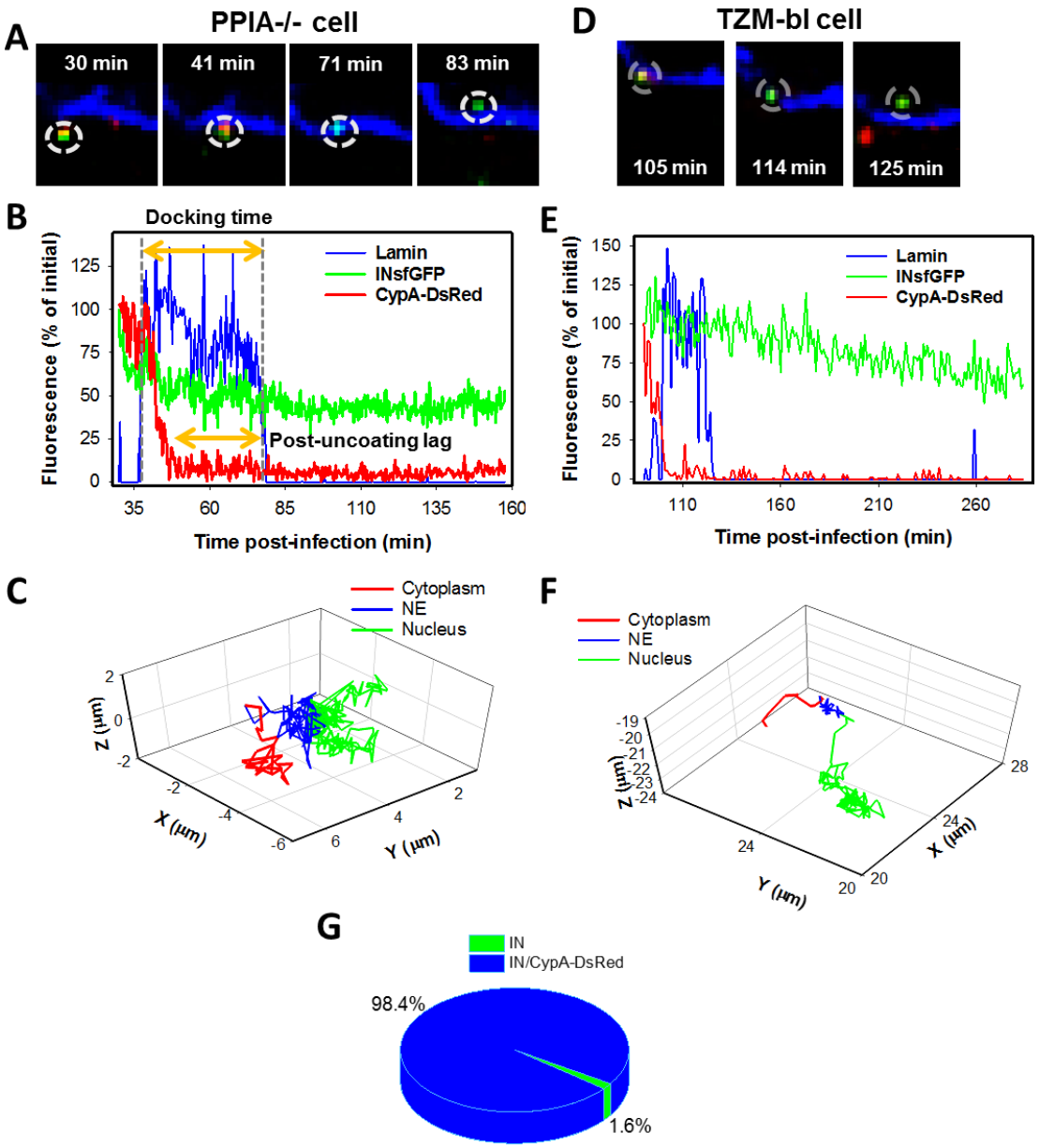

Figure 5. Related to Figure 4. Uncoating at the nuclear pore is a pre-requisite for HIV-1 nuclear entry. Single HIV-1 uncoating and nuclear entry in EBFP2-Lamin expressing **(A-C)** PPIA-/- or TZM-bl **(D-F)** cells. Images were acquired every 20 sec in (A-C) and 1min in (D-F). Time-lapse images of single particle uncoating and nuclear entry in PPIA-/- and TZM-bl cells are shown in (A) and (D), respectively; the fluorescent intensity traces are shown in (B) and (E); and single particle trajectories are shown in (C) and (F). (**G**) A total of 187 single HIV-1 uncoating/nuclear entry events were observed in TZM-bl and PPIA-/- cells and only 3 events corresponded to INsfGFP lacking detectable CypA-DsRed.

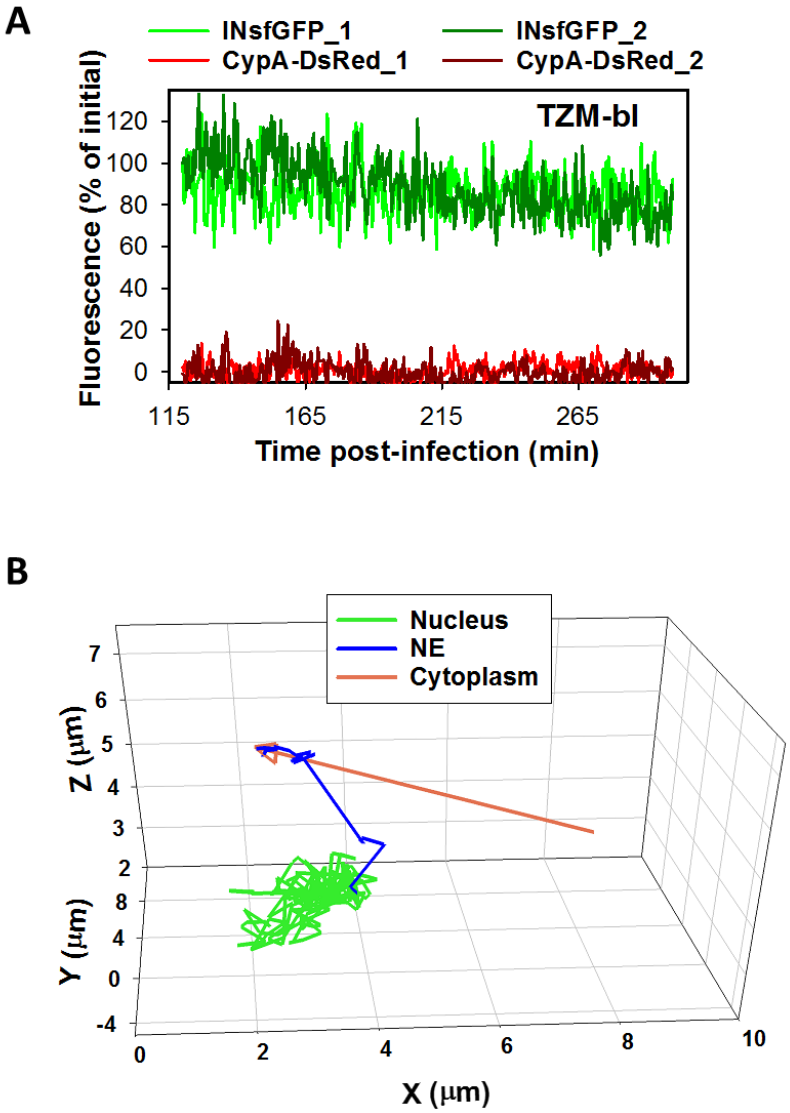

Figure S6. Related to Figure 5. HIV-1 RTC/PICs are stable in the nucleus. (A) Representative fluorescence intensity traces of two representative nuclear HIV-1 INsfGFP complexes that uncoated (lost CypA-DsRed) at the nuclear membrane prior to nuclear import. The CypA-DsRed signal is close to background. Images were acquired every 20 sec. The fluorescence intensities were normalized to the initial INsfGFP and to the mean CypA-DsRed signal of cytosolic cores, respectively, after background subtraction. (**B**) Single particle trajectory of a long-survived nuclear IN/CypA-DsRed complex.

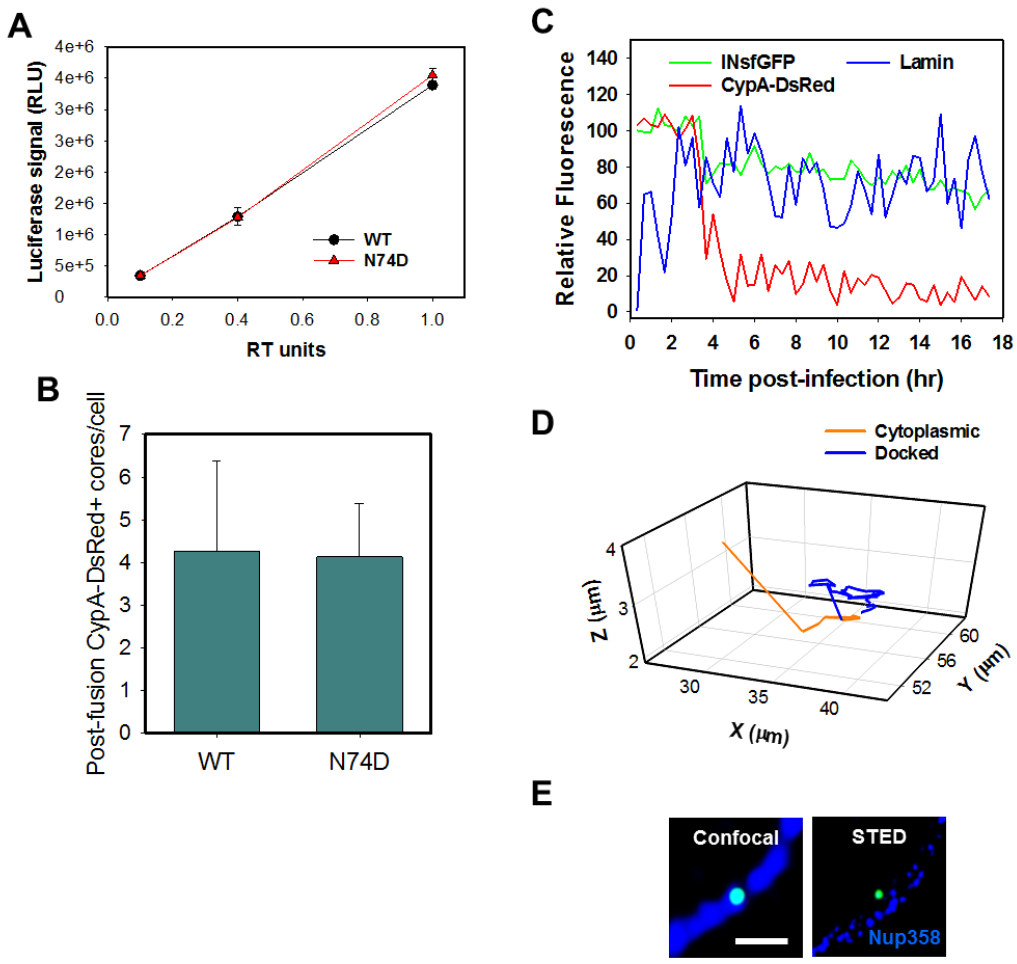

Figure S7. Related to Figure 6. Infectivity, core-stability and nuclear import of mutant CA/N74D viruses in TZM-bl cells. (A) Cells were inoculated with equal RT units (RTU) of the WT and mutant N74D virus supernatant using different dilutions. Infectivity was measured at 48 h.p.i., by luciferase expression (single round infection assay, STAR Methods). A representative triplicate experiment from 3 independent experiments is shown. Error bars = SD. **(B)** HIV-1 core stability in TZM-bl cells was determined by quantifying the number of post-fusion cores, determined as cores shedding CypA-DsRed from INsfGFP complexes in response to CsA (10 µM) application. Cells were inoculated with equal RTU of WT and mutant N74D virus for 3 hours, and cells were moved to the microscope stage maintained at 37°C, 5% CO_2. Live-cell imaging was performed and CsA (10 µM) was added at 15 min after beginning of imaging. The number of INsfGFP/CypA-DsRed double-labeled cores was quantified before and after CsA treatment and the number of post-fusion cores measured by the total number of IN complexes that lost the CypA-DsRed CA marker. Results are average of 2 independent experiments. Error bars are SD. **(C)** Representative fluorescence intensity traces of single N74D/INsfGFP complexes that uncoated (lost CypA-DsRed) at the nuclear membrane of TZM-bl cells and remained associated with the FAP-Lamin. The CypA-DsRed signal loss is slow at the NE (2.5 h). Images were acquired every 20 min starting from 0 h.p.i. (D) Single particle trajectory of a of N74D core in (C). (**E**) Confocal (left) and STED (a representative from 13 complexes visualized in one experiment, right) images of the same intra-nuclear N74D complex labeled with INmNeonGreen. Scale bar 1 µm.