Interaction of the HIV-1 Intasome with Transportin 3 Protein (TNPO3 or TRN-SR2) * §

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Background: TNPO3 is a key cellular factor involved in early steps of HIV-1 replication.

Results: TNPO3 is highly structured, interacts with the HIV-1 intasome by engaging the C-terminal domain of integrase, and does not directly bind capsid tubes.

Conclusion: TNPO3 interacts with HIV-1 intasomes and not capsid cores.

Significance: Our findings aid future genetic analysis to elucidate the role of TNPO3 in HIV-1 replication.

Transportin 3 (TNPO3 or TRN-SR2) has been shown to be an important cellular factor for early steps of lentiviral replication. However, separate studies have implicated distinct mechanisms for TNPO3 either through its interaction with HIV-1 integrase or capsid. Here we have carried out a detailed biophysical characterization of TNPO3 and investigated its interactions with viral proteins. Biophysical analyses including circular dichroism, analytical ultracentrifugation, small-angle x-ray scattering, and homology modeling provide insight into TNPO3 architecture and indicate that it is highly structured and exists in a monomer-dimer equilibrium in solution. In vitro biochemical binding assays argued against meaningful direct interaction between TNPO3 and the capsid cores. Instead, TNPO3 effectively bound to the functional intasome but not to naked viral DNA, suggesting that TNPO3 can directly engage the HIV-1 IN tetramer pre-bound to the cognate DNA. Mass spectrometry-based protein footprinting and site-directed mutagenesis studies have enabled us to map several interacting amino acids in the HIV-1 IN C-terminal domain and the cargo binding domain of TNPO3. Our findings provide important information for future genetic analysis to better understand the role of TNPO3 and its interacting partners for HIV-1 replication.

After gaining entry into a target cell, the HIV-1 core is released in the cytoplasm. The capsid (CA) § protein that forms the outer lattice of the core begins to disassemble as reverse transcriptase converts the viral RNA into a double-stranded DNA copy (1). HIV-1 integrase (IN) binds to the ends of the nascent viral DNA to form the stable synaptic complex (SSC), also known as the intasome (2, 3). This nucleoprotein complex together with a number of cellular and viral proteins forms the preintegration complex (PIC). CA proteins from the partially disrupted CA cores remain associated with the PIC and travel to the nuclear pore. The PIC then traverses the nuclear membrane, and viral DNA is covalently integrated into the host chromosome by transesterification reactions catalyzed by IN. The dimensions of a HIV-1 PIC (~56 nm) significantly exceed the 9-nm size limit required for passive diffusion into the nuclear compartment (4). Therefore, PIC entry into the nucleus depends on an active transport mechanism.

Many studies have attempted to identify key viral and cellular players involved in this process. Several candidates have been proposed including viral determinants such as IN (5), CA (6), matrix (7), and viral protein R (8) as well as the following cellular factors: importin-α (8), importin-β (9, 10), importin 7 (11), TNPO3 (12–14), Nup153, and Nup358 (10, 13–17).

Of these, much attention has recently centered on the cellular protein TNPO3, an importin-β family member that in a RanGTP-dependent manner promotes the nuclear import of serine/arginine rich splicing factors (18). Genome-wide siRNA studies (10, 13) have indicated the importance of TNPO3 to the early stages of HIV-1 replication. Furthermore, Christ et al. (12) have demonstrated that TNPO3 knockdown significantly impaired HIV-1 replication as well as nuclear import but had no effect on MLV replication. Unlike HIV-1, which can infect both dividing and non-dividing cells through the nuclear import of the PIC, the MLV PIC does not traverse through the nuclear pore and instead gains access to chromatin during mitosis. The same study (12) used yeast two-hybrid screens and determined that TNPO3 directly interacts with HIV-1 IN and not with any other retroviral proteins, thus implicating this protein-protein interaction with nuclear import.

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†‡ This article contains supplemental Tables S1 and S2 and Figs. S1–S6.

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3 The abbreviations used are: CA, capsid; IN, integrase; SSC, stable synaptic complex; PIC, preintegration complex; SAXS, small-angle x-ray scattering; NTD, N-terminal domain; CTD, C-terminal domain; CCD, catalytic core domain; Ni-NTA, nickel-nitrilotriacetic acid; NHS, sulfo-N-hydroxysuccinimide; IBC, integrase binding domain.
However, the importance of this direct interaction between HIV-1 IN and TNPO3 has been contested by others (19). For example, it has been shown that both HIV-1 and MLV INs interact with TNPO3 with similar affinities (19, 20). Moreover, a chimeric virus where HIV-1 CA was replaced with its MLV counterpart lost sensitivity to TNPO3, thus highlighting the importance of CA in nuclear import (19). Further argument for the CA dependence on TNPO3 has emerged from the observations that a CA N74D mutant is insensitive to TNPO3 knockdowns (21). This mutant has been identified through a positive selection with respect to a C-terminal-truncated fragment of CPSF6, which effectively restricted HIV replication (21). In this study it was shown that the N-terminal 358-amino acid fragment of CPSF6 binds to wild type CA and not its N74D mutant (21). Full-length CPSF6 contains a SR2 domain at its C terminus (amino acids 527–588) and is thus a potential cargo of TNPO3. Collectively, these findings raised the possibility that TNPO3 effects on HIV-1 replication could be mediated by interaction with the C-terminal SR domain of full-length CPSF6, which in addition could bind CA through interaction with its N-terminal region. However, further studies are necessary to elucidate how CPSF6 and its interaction with TNPO3 could affect lentiviral replication.

A link between HIV-1 entry and its interaction with TNPO3 has also been proposed (20). Infectivity of HIV-1 with the CA N74D substitution but with its native envelope, which mediates the pH-independent uptake, was partly dependent on TNPO3 (20). However, this dependence on the cellular factor was lost when the HIV-1 CA N74D mutant was pseudotyped with vesicular stomatitis virus glycoprotein, which mediates pH-dependent endocytosis (20).

Recent publications have extended the debate about a TNPO3-dependent mechanism in later stages of viral replication by suggesting that TNPO3 affects HIV-1 integration only after PICs enter the nucleus (22–24). One study (24) reported that some CA travels with PICs into the nucleus, after which the TNPO3-RanGTP complex strips the residual CA from the PICs. More recently, it was suggested that TNPO3 could directly interact with the CA cores (23). However, this study used uncleaved CA-nucleocapsid protein and RNA to assemble the CA tubes in vitro, and the control experiments were missing to rule out potential nonspecific charge-charge interactions between TNPO3 and highly polar NC or RNA (23).

In this study we used a battery of biophysical and biochemical techniques to characterize the TNPO3 structure and examine its interactions with either the intasome or CA tubes using an in vitro model system. We have proposed a molecular model of TNPO3 that comprises 20 α-helical HEAT repeats forming a closed ring structure. Binding experiments argue against meaningful direct interactions between TNPO3 and the CA tubes but show instead that TNPO3 readily interact with the HIV-1 intasome. Furthermore, we have mapped the protein-protein interfaces to several HIV-1 IN amino acids in the C-terminal domain and the C-terminal cargo domain of TNPO3. Our findings provide an important framework for future genetic analysis to better understand the role of TNPO3 in HIV-1 biology.

EXPERIMENTAL PROCEDURES

Expression Plasmids—HIV-1 IN clones included the following: full-length, N-terminal domain (NTD) (amino acids 1–50), catalytic core domain (CCD) (amino acids 50–212 with the F185K substitution), and C-terminal domain (CTD) (amino acids 213–288 with the C280S substitution), all with a cleavable N-terminal hexahistidine tag. FLAG-tagged IN was cloned as previously described (25). The TNPO3 expression vector was created by PCR amplification of tnpo3 from a GST-tagged vector (pGEX) containing tnpo3 with the addition of an NdeI and BamHI sites and cloned into pET15b to generate an N-terminal hexahistidine tag. The pET3A expression vector containing full-length HIV-1 CA sequence was a gift from Alan Rein (NCI-Frederick, National Institutes of Health). Site-directed mutants for full-length IN, IN CTD, TNPO3, and CA were created using QuikChange XL kit (Agilent Technologies) according to the manufacturer’s recommendations. LEDGF/p75 was cloned with a hexahistidine tag as described previously (26).

Purification of Recombinant Proteins—Full-length IN was purified as described previously (27, 28). Slight alterations to the protocol included: substitution of 50 mM HEPES, pH 7.5 for 50 mM Tris-HCl, pH 7.4, and the addition of 5 mM β-mercaptoethanol in all buffers, and a 5-ml nickel-nitritoltriacetic acid (Ni-NTA) column (GE Healthcare) was employed with a linear gradient on an AKTA Purifier10 FPLC (GE Healthcare) in lieu of a batch elution. Partially purified protein was either subjected to thrombin digestion or directly loaded onto a 5-ml HiTrap Heparin column (GE Healthcare). The three domains of IN as well as CTD mutants were purified similarly to methods previously described (29). FLAG-tagged IN was purified as described in Kessl et al. (25). Hexahistidine-tagged LEDGF/p75 was purified as previously described (26, 28).

TNPO3 was expressed in Escherichia coli Rosetta cells (Novagen) with a 500 μg isopropyl 1-thio-β-D-galactopyranoside induction for 4 h after an initial A600 of 0.6 was achieved. Cell pellets were resuspended in 50 mM HEPES, pH 7.4, 250 mM NaCl, 2 mM DTT, sonicated, and centrifuged at 20,000 × g for 1 h to remove insoluble material. The lysate was then purified with a Ni-NTA column (GE Healthcare) using a linear gradient of 15 to 500 mM imidazole (in the same buffer). This was followed with a final purification step by isocratic elution from a HiLoad 16/60 Superdex 200 prep grade column (GE Healthcare) in 50 mM HEPES, pH 7.4, 150 mM NaCl, and 2 mM DTT.

Wild type (wt) and N74D CA was purified as previously described (30). Full-length LEDGF/p75 and LEDGF integrase binding domain (IBD) were obtained according to the reported procedures (28). All proteins were checked for purity by SDS-PAGE, quantified by UV absorbance, and stored in 10% glycerol at −80 °C until used.

Preparation of SSC—SSCs were prepared as previously described using 400 nM IN with ~20% forming the SSC (31). SSCs were then mixed with purified 4 μM TNPO3 and allowed to bind to 1 h at 4 °C. Mixtures were then passed through PCR Kleen Spin Columns (Bio-Rad) as described for LEDGF/p75 binding to SSCs (31). The resulting flow-through material was then analyzed by SDS-PAGE and Western blot using anti-hexahistidine (anti-HIS) and anti-IN antibodies (HIV-1 IN mono-
clonal antibody (8G4) from NIH AIDS Research and Reference Reagent Program). The Western blot standard was Magic Mark XP (Invitrogen).

**Pulldown Assays**—FLAG-tagged IN and Anti-FLAG M2 Affinity Gel (Sigma) were used to pull down hexahistidine-tagged TNPO3 or full-length LEDGF/p75. The protocol was followed as previously described (32). After washing the beads, the proteins were extracted from the beads by NuPAGE lithium dodecyl sulfate sample buffer (Invitrogen) and subjected to SDS-PAGE. Nickel affinity pull-down assays using Ni-NTA beads (GE Healthcare) were also done with His-tagged TNPO3 and untagged IN, IN domains, and LEDGF/p40. Resulting pull-down assays were extracted from the beads using NuPAGE lithium dodecyl sulfate sample buffer (Invitrogen), subjected to SDS-PAGE analysis, and visualized by Coomassie staining. Full-length IN pull-down assays were also visualized via Western blotting with the anti-IN antibody (8G4).

CA tubes were formed by the addition of equal volume of a buffer containing 25 mM sodium phosphate buffer, pH 7.5, and 4 mM NaCl to purified 20 mg/ml wt or N74D CA and allowed to polymerize on ice for 2 h (33). Pulldown assays with wt or N74D CA tubes were then performed by centrifugation at 8000 × g after 30 min of a rocking incubation with either TNPO3 or cyclophilin A. Pellets were washed three times with 2 mM NaCl in the 25 mM sodium phosphate buffer, resuspended in NuPAGE lithium dodecyl sulfate sample buffer (Invitrogen), and then run on SDS-PAGE gels and subjected to either Western blot analysis (anti-hexahistidine) or Coomassie staining.

**Circular Dichroism (CD) Spectroscopy**—Purified wild type and mutant TNPO3 proteins were exchanged into buffer containing 50 mM HEPES, pH 7.5, and 250 mM NaCl. Samples (2.5–4 µM) were analyzed in triplicate at 25 °C on a Aviv model 62A DS spectrometer in a buffer containing 10.42 mM NaCl and 4.17 mM sodium phosphate, pH 7.5. Percent of α-helical content was calculated using the K2D3 web server and the mean molar ellipticity (34).

**Mass Spectrometry (MS)-based Protein Footprinting**—Protein-protein interactions were examined using 1 mM sulfo-N-hydroxysuccinimide (NHS)-biotin (Pierce) as described previously (28, 35). IN CTD was modified in the presence or absence of TNPO3. IN CTD was then pulled down as described above using nickel affinity beads. After SDS-PAGE, IN CTD was excised, subjected to in-gel proteolysis by trypsin, and then analyzed by an AXIMA-CFR MALDI-ToF instrument using α-cyano-4-hydroxy-cinnamic acid as the matrix. As a control, the intensities of unmodified CTD tryptic peptides were used to compare across samples.

**Analytical Ultracentrifugation**—Experiments were performed in 50 mM HEPES, pH 7.5, 250 mM NaCl, and 5 mM DTT. Values for the partial buoyant density of the protein (ρ buoy), 0.7499 g/cm³, solvent density (ρ, 1.01 g/ml), and solvent viscosity (η, 0.010 poise) were calculated from buffer composition using the program SEDNTERP (36).

**Sedimentation velocity ultracentrifugation experiments** were performed at 4 °C with an XL-A analytical ultracentrifuge (Beckman-Coulter) and a TiAn60 rotor with two-channel char-coal-filled epon centerpieces and quartz windows. Complete sedimentation velocity profiles were collected every 30 s for 200 boundaries at 45,000 rpm. Data were fit using the c(s) distribution model of the Lamm equation as implemented in the program SEDFIT (37). After optimizing meniscus position and fitting limits, the sedimentation coefficients (s) and best-fit frictional ratios (f/f0) were determined by iterative least squares analysis.

Sedimentation equilibrium data were collected at 4 °C with detection at 280 nm for 5.4, 8.5, and 10.4 µM samples. Analysis was carried out using global fits to all concentrations with data acquired at 8,000, 10,000, and 12,000 rpm using the program SEDPHAT (38) with strict mass conservation. An estimated error for the equilibrium constant was determined from a 1000-iteration Monte Carlo simulation, as implemented in SEDPHAT.

**Size-exclusion Chromatography and Multiangle Light Scattering**—Experiments were performed with a Superdex 200 10/300 GL column (GE Healthcare) at 0.5 ml/min at room temperature in 50 mM HEPES, pH 7.5, 250 mM NaCl, and 5 mM DTT. The column was calibrated using the following proteins (Bio-Rad): thyroglobulin (670 kDa, resolution factor (Rs) = 85 Å), γ-globulin (158 kDa, Rs = 52.2 Å), ovalbumin (44 kDa, Rs = 30.5 Å), myoglobin (17 kDa, Rs = 20.8 Å), and Vitamin B12 (1,350 daltons). Blue dextran (Sigma) was used to define the void volume of the column.

Absolute molecular weights of the proteins studied were determined using multiangle light scattering coupled in-line with size-exclusion chromatography. Light scattering from the column eluant was recorded at 16 different angles using a DAWN-HELOS MALS detector (Wyatt Technology Corp.) operating at 658 nm. The detectors at different angles were calibrated using the small isotropic scatterer horse heart cytochrome c (Sigma). Protein concentration of the eluant was determined using an in-line Optilab T-rEX Interferometric Refractometer (Wyatt Technology Corp.). The weight-averaged molecular weight of species within defined chromatographic peaks was calculated using the ASTRA software version 6.0 (Wyatt Technology Corp.) by construction of Debye plots (KC/rθ versus sin²[θ/2]) at 1-s data intervals. The weight-averaged molecular weight was then calculated at each point of the chromatographic trace from the Debye plot intercept, and an overall average molecular weight was calculated by averaging across the peak.

**Small-angle X-ray Scattering at the Advanced Light Source Beam Line 12.3.1 (SIBYLS)**—X-ray scattering data were measured at the SIBYLS beam line (12.3.1) at the Advanced Light Source (ALS, Berkeley, CA) (39). The forward scattering from the samples studied was automatically reduced using custom software to provide one-dimensional intensity profiles as a function of Q (which is equal to 4πsinθ/λ, where 2θ is the scattering angle) in units of Å⁻¹. Accessible scattering was recorded in the range of 0.010 < Q < 0.35 Å⁻¹. Samples were dialyzed in 50 mM HEPES, pH 7.5, 250 mM NaCl, 2% glycerol, and 5 mM DTT at 4 °C and centrifuged at 3000 rpm for 10 min at 4 °C before data were recorded. Data were collected using a 96-well plate handling sample robot, as previously described (39), with three concentrations: 12, 8, and 5 mg/ml TNPO3 in a total
volume of 24 μl. All samples were characterized with 0.5, 1, and 6 s exposures at 20 °C at a wavelength of 1 Å. Scattering from a matching buffer solution was subtracted from the data and corrected for the incident intensity of x-rays. Replicate exposures were examined carefully for evidence of radiation damage by Guinier analysis and Kratky plot analysis. Silver behenate powder was used to locate the beam center and to calibrate the sample-to-detector distance.

Small-angle X-ray Scattering (SAXS) Data Analysis—All of the preparations analyzed were monodisperse, as indicated by linearity in the Guinier region of the scattering data and agreement of the I(0) and Rg values determined with inverse Fourier transform analysis by the programs GNOM (41). Molecular mass as derived from transform analysis by the programs GNOM (41). Molecular linearity in the Guinier region of the scattering data and agreement of the preparations analyzed were monodisperse, as indicated by the apparent shape of the particle and improvement in analyses and then repeated assuming 2-fold symmetry as justified by the apparent shape of the particle and improvement in the final χ and normalized spatial discrepancy criterion. The models resulting from the independent runs were superimposed by the program SUPCOMB based on normalized spatial discrepancy criterion. The 10 independent reconstructions were then averaged and filtered to a final consensus model using the DAMAVER suite of programs (48). Consensus models obtained by DAMMIN and GASBOR approaches yielded similar results.

Shape Reconstruction—Low resolution shapes were determined from solution scattering data using the programs DAMMIN (43) and GASBOR (44). Bead models were visualized in PyMOL (45) or converted to volumetric envelopes using SITUS (46) and visualized using UCSF Chimera (47). With GASBOR, the number of dummy residues used in shape reconstruction is prescribed by the user, requiring an understanding of the composition of the particle being modeled. Ten independent calculations were performed for each data set using default parameters. Initially, no symmetry constraints were applied in all analyses and then repeated assuming 2-fold symmetry as justified by the apparent shape of the particle and improvement in the final χ and normalized spatial discrepancy criterion. The models resulting from the independent runs were superimposed by the program SUPCOMB based on normalized spatial discrepancy criterion. The 10 independent reconstructions were then averaged and filtered to a final consensus model using the DAMAVER suite of programs (48). Consensus models obtained by DAMMIN and GASBOR approaches yielded similar results.

Homology Model of Human Transportin—The primary sequence of TNPO3 (Q9Y5L0) was used as a query sequence for five iterations of PSI-BLAST (49) searching of the NCBI RefSeq database (50). A large number of homologs were identified; the best scoring protein from this query for which an atomic structure has been determined is Importin-13 (PDB 2X19, Chain B (51)), with an initial E score of 2e−32 and 24% sequence identity. Three crystal structures of Importin-13 are currently available in the PDB, each in complex with a different protein: PDB codes 2XWU, 2X19, and 2X1G.

TNPO3 sequence was threaded onto the 2X19 Importin-13 template structure using the program MODELLER 9v8 (52). The resulting model was gradually relaxed by energy minimization in a solvated environment using the programs VMD (53) and NAMD (54) using CHARMM42 force fields. Inspection of the resulting model revealed the need for manual rebuilding of the loop 841–855; the rebuilt loop was subsequently minimized in vacuo. The overall quality of this minimized model was evaluated using the MOLPROBITY server (55). 98.6% of the residues resided in the allowable regions of a Ramachandran plot and showed generally good stereochemical properties. The model was rendered using the program PyMOL (45).

RESULTS

The importance of TNPO3 for HIV-1 replication has been well established (10, 12–14, 19, 22–24, 56). However, previous reports differ in implicating either HIV-1 IN or CA as direct binding partners of TNPO3 (12, 19, 22, 23). Because these studies examined the interactions employing different experimental approaches, we compared TNPO3 interactions with HIV-1 IN and CA in parallel experiments using purified recombinant proteins and pulldown assays. Although purified recombinant CA is monomeric, at elevated NaCl concentrations CA assembles into tubes that closely resemble the CA cores from infected cells (30, 57). Using these assembled CA tubes under previously defined conditions (33), we first confirmed their interactions with a cognate cellular binding partner cyclophilin A (CypA; Fig. 1A). These assay conditions were next applied to probing interactions between CA tubes and increasing concentrations (1–5 μM) of TNPO3. The data in Fig. 1B show trace binding (~3%) of TNPO3 to CA tubes at the highest concentration tested (5 μM). In parallel reactions we examined binding by the N74D CA mutant (Fig. 1C) as this substitution has been shown to relieve the dependence of HIV replication on TNPO3 (21). The results in Fig. 1A, C and E, show similarly minimal levels (~3%) of TNPO3 binding with wild type and the N74D mutant CAs. We next probed the interaction between HIV-1 IN and TNPO3 using the same concentration range of the cellular protein. The data in Fig. 1A, D and E, show strong binding, with ~47% of 2 μM TNPO3 pulled down by 2 μM HIV-1 IN under these conditions. We did not observe a significant increase of TNPO3 pulldown levels with increasing concentrations (2–10 μM) of the cellular protein indicating that these protein concentrations are well above the Kd value for the IN–TNPO3 interaction. Thus, our findings are consistent with the previous report (20) of the Kd value for the IN–TNPO3 binding being in the low nM range. Taken together, our results argue that TNPO3 can effectively interact with HIV-1 IN and not with CA tubes in vitro.

In infected cells HIV-1, IN functions in the context of a large nucleoprotein assembly termed the PIC, in which a tetramer of IN is stably bound to the viral DNA ends. Furthermore, a number of viral and cellular proteins associate with PICs. Of these, LEDGF/p75 is a principal cellular binding partner of IN. Therefore, it is important to examine how viral DNA and LEDGF/p75 affect the IN interaction with TNPO3. We previously reported a method for assembly of the SSC in vitro (31) that closely mimics IN–viral DNA interaction in vivo. Schematics for the experimental approach for the SSC binding to TNPO3 and corresponding data are given in Fig. 2. A and B. These results show that TNPO3 selectively interacted with the SSC and not with free DNA (compare lanes 6 and 7 in Fig. 2B). These experiments
Our next goal was to map individual HIV-1 IN domains interacting with TNPO3. HIV-1 IN is composed of three structurally and functionally distinct domains: an NTD that contains a Zn²⁺ finger motif (58), the CCD that contains the acidic DDE triad that coordinates catalytic Mg²⁺ ions (59, 60), and a CTD that is highly basic and exhibits an Src homology-3-like-fold (61). All three domains interact with viral DNA (62, 63). Studies with prototype foamy virus IN indicate that the CCDs and NTDs from separate subunits provide key interfaces for functional multimerization of IN (62). Furthermore, these two protein domains directly coordinate binding to LEDGF/p75 (28, 64). In contrast the CTD does not interact with LEDGF/p75 (28). The data depicted in Fig. 3 reveal strong interactions between TNPO3 and the isolated CTD. No interaction was detected with the CCD, and only minimal interaction was observed with the NTD (Fig. 3). These experiments suggested that HIV-1 IN CTD contains the primary binding site for TNPO3.

Our subsequent experiments focused on mapping CTD residues responsible for binding to TNPO3. For this we used a MS-based protein footprinting technology that enabled us to map interacting amino acids based on comparison of their chemical reactivity in the free protein versus the protein-protein complex. The full list of the interacting amino acids identified by MALDI-ToF is shown in supplemental Table S2, and Fig. 4 depicts representative segments of the mass spectra for the isolated CTD. The peak (containing a fragment of amino acids from 264–269) contained two modified lysine residues (Lys-264 and -266) indicating that these sites are surface-exposed in the CTD. No interaction was detected with the CCD, and only minimal interaction was observed with the NTD (Fig. 3). These experiments suggested that HIV-1 IN CTD contains the primary binding site for TNPO3.

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suggest that the IN tetramer in the context of the SSC can effectively engage TNPO3. We next formed an IN-LEDGF/p75 complex and probed its interaction with TNPO3. The data in Fig. 2C show that even a 5-fold excess of LEDGF/p75 over IN had no affect on the IN-TNPO3 binding. In control experiments TNPO3 failed to directly interact with full-length LEDGF/p75 or LEDGF IBD (Fig. 2D), thus indicating that the IN-TNPO3 interaction is specific.

FIGURE 1. Representative pulldown experiments for the following reactions: wt CA tubes plus recombinant CypA (panel A), wt CA tubes plus TNPO3 (panels B), N74D CA tubes plus TNPO3 (panel C), and FLAG-IN plus TNPO3 (panel D). Panel A is a Western blot using anti-His antibodies. Panels B–D are Coomassie-stained SDS-PAGE gel images. Panels A–D: lane 1 contains molecular weight standards with their masses designated on the side. Panel A, lane 2, control containing only wt CA tubes; increasing concentrations (2, 5, and 10 μM) of CypA were incubated with (lanes 3–5) or without (lanes 6–8) wt CA tubes; lane 9 is a full load of CypA (A). CA tubes were formed using 20 mg/ml protein solution as described under “Experimental Procedures.” Panel B: lane 2, control containing only wt CA tubes; increasing concentrations (2, 5, and 10 μM) of TNPO3 were incubated with (lanes 3–5) or without (lanes 6–8) wt CA tubes; lane 9, 1/10th loads of wt CA and 5 μM TNPO3. Panel C is identical to panel B with the exception that the reactions contained N74D CA instead of wt CA. Panel D: lane 2, control experiment with FLAG-IN (2 μM) incubated with anti-FLAG M2 affinity gel; increasing concentrations (2, 5, and 10 μM) of TNPO3 were incubated with (lanes 3–5) or without (lanes 6–8) FLAG-IN followed by affinity pulldown using anti-FLAG M2 affinity gel. Heavy and light chain FLAG antibodies are indicated by stars; lane 9: 5 μM load of TNPO3. Panel E is the graphic representation of the relative binding seen in the first four panels with the S.D. of triplicate runs indicated.
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FIGURE 2. TNPO3 binding to the IN-viral DNA (panels A and B) and IN-LEDGF/p75 complexes (panel C). Panel A shows the schematic of TNPO3 binding to the SSC; only TNPO3 bound to the SSC makes it through the spin size-exclusion column, whereas the unbound protein is retained in the matrix. Panel B is SDS-PAGE analysis of the flow through of the size-exclusion columns runs. The protein bands were visualized by a Western blot using a mixture of anti-HIS and anti-IN antibodies. Lanes 1 and 2 contain 1/10th loads of TNPO3 and IN, respectively. Lanes 3, the molecular weight standards (sizes are indicated on the side). Lanes 4 – 8 contain the flow-through material. Lane 4, IN alone; lane 5, IN plus viral DNA or the SSC; lane 6, the SSC plus TNPO3; lane 7, viral DNA plus TNPO3; lane 8, TNPO3 alone. Panel C is an SDS-PAGE gel image of TNPO3 interactions with the preformed complex of the FLAG-IN with LEDGF/p75. Lane 1 contains the molecular weight standards, and lanes 2–7 are FLAG affinity bead pulldown assays. Lane 2, control pulldown assay with only LEDGF/p75 and TNPO3 (both 5 µM). In lane 3–7, 2 µM FLAG-IN was preincubated with increasing LEDGF/p75 concentrations (0, 1.25, 2, 5, and 10 µM, respectively), and then 10 µM TNPO3 was added to the reaction. Lanes 8–10 contain 1/5th loads of IN, LEDGF/p75, and TNPO3 in that order. Heavy and light chain FLAG antibodies are indicated by stars. Panel D is an SDS-PAGE gel of nickel affinity pulldown assays using His-TNPO3. Lanes 1 and 5 are molecular mass standards, and lanes 2–4 are 1/2 loads of His-TNPO3, LEDGF/IBD, and LEDGF/p75, respectively. Lanes 6–10 are nickel affinity pulldown assays with 5 µM HIS-TNPO3 (lanes 6, 8, and 10) and 20 µM tag-free LEDGF/IBD (lanes 7 and 8) or 20 µM tag-free LEDGF/p75 (lanes 9 and 10).

FIGURE 3. Representative SDS-PAGE gel images of nickel affinity pulldown assays of HIV-1 IN domains with His-TNPO3. Lanes 1 and 6 are molecular mass standards, and lanes 2–5 are 1/2 loads of His-TNPO3, IN CTD, IN CCD, and IN NTD, respectively. Lanes 7–13 are nickel affinity pulldown assays with 5 µM His-TNPO3 (lanes 7, 9, 11, and 13) and 20 µM untagged CTD (lanes 8 and 9), 20 µM untagged CCD (lanes 10 and 11), or 20 µM untagged NTD (lanes 12 and 13).

ification in free CTD and the CTD-TNPO3 complex (compare right panels of A and B in Fig. 4). In general, we mapped several reactive lysines in the free CTD (Lys-219, -240, -264, -266, and -273). Of these, only the Lys-264/266 peak was protected in the CTD-TNPO3 complex whereas other lysines were modified to a similar extent in the free protein and protein-protein complex.

We next used site-directed mutagenesis to validate residues important for the CTD-TNPO3 interaction. The data presented in Fig. 5, A and B, agree well with MS-based footprinting (Fig. 4) showing the significance of Lys-264 but not Lys-266 for the CTD-TNPO3 interactions. In addition, mutagenesis experiments revealed the importance of Arg-262 and Arg-263 for the interaction. Taken together, the protein footprinting and site-directed mutagenesis data suggest that Arg-262, Arg-263, and Lys-264 directly interact with TNPO3. The mutagenesis studies were extended further to probe interactions between full-length IN and TNPO3. Fig. 5D shows that the R262E/R263E/K264E substitution significantly compromised the IN-TNPO3 binding. The triple mutant was also defective for SSC formation (Fig. 5E) but maintained wild type levels of binding with LEDGF/p75 (Fig. 5F). As an additional confirmation that these substitutions did not affect the overall IN structure, we performed protein footprinting analysis of the surface-exposed lysine residues using NHS-biotin. As expected, the substitutions of the Arg and Lys residues affected the trypsin cleavage pattern of this protein segment. However, no significant differences were observed in the modification profiles of the other Lys residues when the triple mutant was compared with wt IN (data not shown) indicating that the R262E/R263E/K264E substitutions did not grossly alter the protein structure.

To explain the above experimental observations, we analyzed a molecular model of HIV-1 intasome or the SSC bound to LEDGF IBD (Ref. 31; see also supplemental Fig. S1). This model is based on the crystal structure of the prototype foamy virus intasome (62) and our FRET analysis of the HIV-1 SSC and its interaction with LEDGF/p75 (31). The assembly of the SSC requires a tetramer of IN with two subunits tightly interacting with viral DNA and other two subunits providing a supporting...
**FIGURE 4.** RES-based protein footprinting of the CTD interaction with TNPO3. Representative sections of tryptic-digested peptide peaks from the CTD are shown. In panel A, CTD was treated with 1 mM NHS-biotin. Biot, biotin. Panel B, the CTD was preincubated with TNPO3 and then subjected to treatment with 1 mM NHS-biotin. Panel C contained untreated CTD. Start and end amino acid numbers of the detected peptide peaks are indicated. The Lys residues modified with NHS-biotin are indicated in brackets.

**FIGURE 5.** Analysis of HIV-1 IN mutants. Representative SDS-PAGE gel images of nickel affinity pulldown assays of the CTD mutants with His-TNPO3 are shown in panel A. Lanes 1 and 2 contain molecular weight standards and 1/2 load of TNPO3, respectively. Lanes 3–18 contain pulldown assays of 20 µM CTD mutants (as indicated above the gel) and the same mutant plus 5 µM His-tagged TNPO3 in alternating fashion. Panel B shows a Coomassie-stained gel of 1/5th load of the CTD mutants in the same order as panel A with molecular weight standards shown in lane 1. Panel C shows the graphic representation of triplicate runs of the relative binding amounts seen in panel A with the S.D. indicated. Panel D represents a nickel affinity pulldown of full-length IN mutant (R262E/R263E/K264E) by His-TNPO3. The Western blot (1/20th of the total reaction) was developed with anti-IN with 5 µM wild type IN (lanes 2 and 3) compared with 5 µM mutant IN in lanes 4 and 5. 1.25 µM His-TNPO3 was added to lanes 3 and 5. Molecular weight standards are shown in lanes 1 and 6. Panel E examines the ability of wild type IN (lane 6) and the R262E/R263E/K264E mutant (lane 7) to form the SSC. Lanes 1 and 2 contain 1/20th loads of IN and mutant, respectively, and lanes 4–7 contain the flow-through of the spin size-exclusion column (see Fig. 2A). Lane 3 contains the molecular mass standards. In lanes 4 and 5 wild type and the mutant INs, respectively, were subjected to the spin size-exclusion separation. In lanes 6 and 7 viral DNA was added to wild type and the mutant INs, respectively, and the complexes were then subjected to a spin size-exclusion chromatography. IN associated with the SSC was detected by Western blot using anti-HIS antibody. Panel F shows a representative SDS-PAGE gel image of nickel affinity pulldown assays of wild type and the mutant (R262E/R263E/K264E) INs with His-LEDGF/p75. Lane 1, molecular mass marker; lanes 2 and 3, tag-free wild type and the mutant INs incubated with nickel beads; lane 4, His-LEDGF/p75; lane 5, wt IN plus His-LEDGF/p75; lane 6, R262/3/4E IN plus His-LEDGF/p75.
Although there are no available experimental structures of TNPO3 alone as a step toward better understanding the structural basis for its interaction with the intasome. Such a model is consistent with our experimental observations that the SSC directly interacts with TNPO3 (Fig. 2B) and that the triple substitution affects pleiotropic effects compromising the IN binding to both the viral DNA and TNPO3 (Fig. 5, D and E). Furthermore, the model for the SSC-LEDGF IBD complex (supplemental Fig. S1) shows that the CTDs in the supporting subunits remain surface-exposed upon binding of LEDGF IBD (shown in gray) to the SSC and could thus directly engage TNPO3. Accordingly, we found that the preformed IN-LEDGF:p75 complex interacted with TNPO3 (Fig. 2C), and the triple substitutions in the CTD of full-length IN did not affect IN-LEDGF:p75 binding (Fig. 5F).

Our next goal was to investigate the structural properties of TNPO3 alone as a step toward better understanding the structural basis for its interaction with the intasome. Although there are no available experimental structures of TNPO3 to date, structures of related importin-β family members are available. A PSI-BLAST (49) search of the NCBI RefSeq database (50) identifies Importin-13 (51) as the closest homolog for which an atomic structure has been determined experimentally (24% sequence identity, supplemental Fig. S2). Using this structure as a template, we generated a homology model of TNPO3 (Fig. 6A). With this level of sequence identity (the “twilight zone” in homology modeling), there will be significant local and gap errors with individual pairwise alignments; however, it is expected that such a model will still correctly capture the gross quaternary features (65).

Members of this family all share a topology that features stacked HEAT domain repeats containing paired antiparallel α-helices connected by flexible loops (66). To investigate the secondary structure of TNPO3 and to confirm its predicted α-helical character, we employed CD spectroscopy. The data in Fig. 6B show that recombinant TNPO3 features a high (~58%) α-helical content (versus 70% predicted from the homology model and 68% for Importin-13). Thus, we conclude the secondary structure of TNPO3 is similar to that of other importin-β family members and is concordant with our homology model (Fig. 6A).

To better understand the solution behavior of TNPO3, we next performed size-exclusion chromatography in-line with multiangle light scattering and sedimentation velocity. These approaches indicated the presence of both monomers and dimers in solution at the concentrations tested (Fig. 6, C and D, and supplemental Fig. S3). We extended our analysis to determine the $k_d$ for dimerization by sedimentation equilibrium analysis. Importin-β from mouse has been reported to behave as a monomer in solution (67). In contrast, sedimentation equilibrium analysis (Fig. 6E) indicates that TNPO3 is best described as existing in a monomer-dimer equilibrium in solution, with a $k_d$ for dimerization of 8.3 μM ± 0.99.

SAXS can provide insight into the shape and size of macromolecules as they exist in solution and allows for the reconstruction of structure at moderate resolution (~15 Å). SAXS analyses were performed at concentrations well in excess of the determined $k_d$ for dimerization to ensure analysis of a single species dimer in solution (Fig. 7A). At the concentrations examined, I(0) analysis confirms the dimeric mass of TNPO3 (supplemental Table 1). Reconstruction of the TNPO3 dimeric structure at low resolution using the program GASBOR (44) with P2 symmetry constraints yielded a bilobal prolate “saddle-shaped” ellipsoid. The final averaged and filtered shape had dimensions 97.5 × 120.0 × 68.9 Å (Fig. 7B and supplemental Fig. S4); similar results were obtained with the program DAMMIN (43) (data not shown). By HYDROPRO (68) analysis, the averaged unfiltered shape has a calculated $R_g$ of 48 Å and an s value of 6.4, in concordance with SAXS measurements ($R_g = 48$ Å) and sedimentation velocity measurements (s of 6.3 for the dimer peak). The excluded hydrated volume for the final SAXS reconstruction (313,800 Å³) is largely consistent with the predicted volume of a well-folded dimer based only on primary sequence (264,874 Å³).

In good concordance with our understanding of the association properties, secondary structure, and our homology model of the TNPO3 monomer, two TNPO3 model monomers in the closed ring configuration could be readily docked into the GASBOR reconstruction (supplemental Fig. S5). This modeling exercise requires that both monomers associate in such a way that would preclude cargo binding, as indicated in available Importin-13 crystal structures (66). However, the concentrations at which a TNPO3 dimer predominates are arguably well above physiologically relevant levels, whereas the $k_d$ for the IN-TNPO3 interaction is ~36 nM (20). Thus, although the in vitro hydrodynamic data validate the homology model and describe the quaternary structure of the dimer, we predict that monomeric TNPO3 binds the intasome.

Our next goal was to map IN binding sites on TNPO3. Application of MS-based protein footprinting revealed several surface lysine residues (Lys-355, -366, -594, -651, -877, -881, and -909) in free TNPO3 and further confirmed the validity of the proposed molecular model. However, these residues were not protected from chemical modification in the presence of the CTD or full-length IN, indicating that they are not involved in direct protein-protein interactions. Therefore, we next exploited the available structural and mutagenesis data for Importin-13 (both human and Drosophila forms) as well as the more divergent transportin-1 and interactions with their cargoes to predict potential IN binding sites in TNPO3. For example, TNPO3 Glu-304, Glu-391, and Arg-402 were selected because corresponding residues in Importin-13 have been shown to bind to Ubc9 (66), TNPO3 Glu-392 and Arg-400 were mutated because the corresponding residues in Importin-13 have been shown to bind to Mago-Y14 (51).
The data in Fig. 8 show that E304R and E391R/E392R substitutions had very little affect on the IN-TNPO3 interaction. In contrast, substitutions of R400E/R402E and Q761E/R762E significantly impaired the IN binding to TNPO3. As a control, CD experiments revealed that wild type and the two reduced binding mutants of TNPO3 had very similar secondary structures, indicating that these substitutions did not result in gross loss of structure of the protein. Therefore, our results indicate that the C-terminal cargo domain of TNPO3 directly interacts with IN. The model presented in supplemental Fig. S6 shows that the CTD of IN could indeed engage the C-terminal segment of TNPO3.
TNPO3 Does Not Interact Directly with CA Tubes in Vitro—

Several studies have implicated CA as a primary determinant of HIV-1 dependence on TNPO3 (19, 21, 70). HIV-1 CA containing an N74D substitution circumvented the inhibition of HIV-1 infection, which was seen during TNPO3 knockdown (19, 21, 70). However, it is not clear from these studies whether CA and TNPO3 interact directly or if their interactions are mediated by another factor(s). The recent report (23) has suggested that TNPO3 directly binds the CA cores. However, the binding experiments were conducted using uncleaved CA-NC protein and RNA (23). Although the outer surface of correctly assembled CA-NC does resemble the CA cores, these assembly conditions yield heterogeneous mixtures, and one cannot exclude a possibility of potential low affinity interactions of TNPO3 with highly polar NC or RNA. In addition, the observed interactions between TNPO3 and the tubes assembled with CA-NC and RNA required extremely high quantities of TNPO3, allowing for the potential for lower affinity charge-charge interactions between TNPO3 and NC or RNA to be seen (23), and the control experiments were not reported to rule out this possibility. Furthermore, the authors observed only a very modest difference in binding affinity between wild type and N74D CA-NC (~1.5-fold), whereas in the context of HIV-1 replication in TNPO3 knockdown cells they observed ~12-fold differences between wild type and the N74D CA mutant viruses (23). In this work we used highly purified CA without the addition of NC or RNA. First, the monomeric CA protein failed to exhibit any interaction with TNPO3 (data not shown). We next assembled CA tubes under defined conditions (33) and verified their interactions with cognate CypA (Fig. 1). Under the same assay conditions, only residual amounts of TNPO3 associated with the CA tubes. Moreover, the levels of TNPO3 interactions with wild type and N74D CA were very similar. Taken together, our results argue against meaningful direct interactions between TNPO3 and CA.

How do our findings reconcile with the observation (21) that HIV-1 containing either wild type or N74D mutant CA exhibit different nuclear import phenotypes in TNPO3 knockdown cells? One possibility is that the N74D substitution alters interplay between CA and another factor, which in turn directly or indirectly links this protein-protein complex to TNPO3. For example, CPSF-6 has been shown to interact with wild type and not N74D CA (21, 70). Furthermore, a recent study (70) has mapped a small CPSF-6 segment of amino acids 314–322 that directly engages wild type CA. At its C terminus (amino acids 527–588) CPSF-6 contains an SR domain, which is a potential discriminator between TNPO3 (71) and, therefore, likely to mediate the

DISCUSSION

TNPO3 and CA...
nuclear import of RNA processing proteins. Therefore, CPSF-6 could potentially act as bifunctional tether, with amino acids 314–322 interacting with CA and the SR domain engaging TNPO3. However, further studies are necessary to test whether such a ternary complex is formed in infected cells. Alternatively, TNPO3 knockdown could lead to relocation of CPSF-6 from its normal location in the nucleus to the cytoplasm, where CPSF-6 could directly engage the CA cores and interfere with its uncoating or docking to the nuclear pores. In support of this premise, the CPSF6–358 protein that lacks the SR domain has been shown to localize in the cytoplasm and potently inhibit HIV-1 replication through direct interaction with CA (21). Furthermore, HIV-1 acquired resistance to CPSF6–358 through the N74D CA substitution. Taken together, the current knowledge does not allow us to delineate how CPSF-6 modulates the HIV-1 nuclear import. Therefore, further studies are warranted to probe the mechanisms involving CPSF-6 as well as to identify other potential factors mediating the interplay between TNPO3 and CA.

The HIV-1 Intasome Directly Interacts with the TNPO3 in Vitro—Yeast two-hybrid and pulldown assays (12) have shown that HIV-1 IN directly interacts with TNPO3. Using MS-based footprinting and site-directed mutagenesis, we have identified IN CTD residues R262E/R263E/K264E as being important for this protein-protein interaction. Although the yeast two-hybrid screens (12) pointed to the significance of HIV-1 IN CCD for the interaction with TNPO3, the absolute majority of their hits included almost all of the CTDs including the residues we have indentified. In these studies we have used stringent assay conditions (for example, the pulldown reactions contained 500 mM NaCl) to identify primary binding sites of TNPO3 in HIV-1 IN. These reaction conditions have revealed the HIV-1 IN CTD as the principal interacting partner of TNPO3. At the same time, our data do not exclude a possibility that TNPO3 could establish lower affinity interactions with HIV-1 IN CCD or NTD.

A previous study (72) demonstrated that K215A/K219A and R263A/K264A substitutions severely impair nuclear import of PICs and compromise IN binding to Importin-α3. Furthermore, a more recent report has uncovered the significance of Lys-264 for the IN interaction with an undefined nuclear import factor and HIV-1 integration (73). Although the K264E substitution fully impaired IN CTD binding with TNPO3 (Fig. 5A), the same single site substitution in the context of full-length IN had only a modest effect on the IN-TNPO3 binding (data not shown), and additional substitutions of adjacent Arg-262 and R263 were necessary to compromise the interaction between full-length proteins (Fig. 5D). Thus our and published (72, 73) findings collectively argue for the importance of this IN segment for HIV-1 integration and call for further more detailed genetic analysis of these substitutions during HIV-1 infection. Because the IN R262E/R263E/K264E mutant is defective for interactions with TNPO3, Importin-α3, and viral DNA, the main focus of future genetic studies should be to carefully delineate pleotropic affects arising from these substitutions.

We show for the first time that TNPO3 can affectively bind the functional IN-viral DNA stable synaptic complex in
vitro. Molecular modeling has provided a useful structural context for understanding such interactions. For example, within the functional intasome, where a tetramer of IN is stably bound to the two viral DNA ends, the CTDs in the two IN subunits that serve a supporting role in the intasome are fully accessible to engage TNPO3. In line with these observations, the IN-TNPO3 interaction is not affected by LEDGF/p75 (Fig. 2C), as the latter binds the IN CCD and NTD but not the CTD (28). Taken together, our data suggest that both LEDGF/p75 and TNPO3 could engage the HIV-1 intasome at the same time.

Previous studies (19, 20) have demonstrated that MLV as well as a number of other retroviral INs also strongly interact with TNPO3. Structural similarity between CTDs of different retroviral INs could explain these observations. However, the functional significance of direct interactions between retroviral INs and TNPO3 has been questioned due to the findings that MLV vectors as well as the chimeric HIV-1 virus containing MLV CA are insensitive to TNPO3 knockdown (6, 19). One possible explanation for this is that the differential uncoating rates of MLV and HIV-1 CA cores could differently modulate TNPO3 access to the intasome. For example, it has been observed that HIV-1 CA dissociates from PICs more rapidly than their MLV counterparts (74, 75). Thus, the CA cores could provide masking effects until uncoating, and hence their rates of uncoating could accordingly regulate TNPO3 access to the intasome. Roles of other cellular factors should also be considered. For example, CPSF-6 has been shown to directly interact with HIV-1 but not MLV CA and as discussed above could potentially facilitate the nuclear import of HIV-1 by altering the availability of the intasome. Although these scenarios support the notion that the primary dependence of HIV-1 on TNPO3 is determined by CA, they do not necessarily exclude a possibility that the intasome directly engages TNPO3 at later stage of HIV-1 replication just before its nuclear entry or after the PICs travel to the nucleus.

Recent studies have suggested that the TNPO3 within the nucleus could be important for promoting HIV-1 integration (22–24). A mechanism was proposed whereby some PIC-associated HIV-1 CA enters the nucleus and TNPO3 strips the CA from PICs to promote effective integration (24). If this model gains further support, one could consider a possibility where CA is displaced from PICs by the direct binding of TNPO3 to the intasome rather than CA. Recent reports have also shown that TNPO3 knockdowns reduced HIV-1 integration frequency into gene-dense regions (14). Our findings suggest that TNPO3 could directly associate with the functional nucleoprotein complex and could thus potentially affect the integration site targeting. Again, alternative scenarios could not be excluded, with TNPO3 effects on the integration efficiency and site preference being mediated by other cellular factors. Our mapping of interacting interfaces between HIV-1 IN and TNPO3 provides an important tool for further genetic analysis to delineate the mechanism for HIV dependence on TNPO3.

Structural Characterization of TNPO3—We report the first detailed biophysical characterization of TNPO3. The CD experiments have revealed a highly α-helical secondary structure of TNPO3 pointing to its close resemblance with Importin-13. Similarities between these two proteins are further evident from their primary structure comparisons indicating 25% sequence identity and 44% similarity. Therefore, it was sensible to use the available x-ray crystal structures of Importin-13 (51, 66) for the molecular modeling of TNPO3. Although the free full-length Importin-13 structure is not available, the structures of Importin-13 in its complexes with different cargoes revealed a remarkable conformational flexibility of the protein (51, 66). For example, Importin-13 in the complex with Mago-Y14 adopts an open super-helical conformation (51), whereas the Importin-13-Ubc9 complex formed a closed ring conformation (66). Although it was not possible to analyze exclusive monomers of TNPO3 by SAXS because of self-association, our modeling of the TNPO3 dimer does indicate that at least in the context of a dimer, TNPO3 monomers exist in a closed ring configuration (Fig. 6A), where the N- and C-terminal segments form an interface with each other.

The N-terminal portion of TNPO3 shares the highest degree of amino acid sequence conservation with Importin-13 and other members of the Importin-β family and is likely to directly bind their common regulator RanGTP (76). Indeed many of the key residues identified in Importin-13 as directly binding RanGTP are also present in TNPO3 (51). The C-terminal portions of these karyopherins usually bind their respective cargos (69, 77–83). However, two exceptions have been reported with Ubc9 and parathyroid hormone-related protein interacting with the N-terminal arches of Importin-13 and Importin-β, respectively (40, 66). We used several contact points observed in the Importin-13 co-crystal structures with different cargoes (51, 66) to predict the TNPO3 binding site to HIV-1 IN. Our data suggest the importance of TNPO3 residues Arg-400/Arg-402 and Glu-761/Arg- for binding with HIV-1 IN. These residues are in the inner C-terminal arch facing each other, and the IN CTD can readily be modeled in this region to establish direct contacts with these residues. Based on the size of the IN CTD, it can fit within the cavity formed by the closed ring structure (supplemental Fig. S6).

A previous study mapped several TNPO3 amino acid sites as being important for lentiviral replication (56). The most significant effect on simian immunodeficiency virus nuclear import was observed when the L767A/L768A substitutions were introduced in TNPO3. In our model these residues are located in a loop spanning amino acids 763–775, which immediately follows the α-helix of amino acids 747–762 containing Glu-761/Arg-762. Thus, previous and current studies collectively point to the importance of this segment for the function of TNPO3. However, it remains to be seen whether this TNPO3 segment engages HIV-1 IN or another cargo in infected cells. Another TNPO3 region mapped by the published study implicated the C-terminal 18 amino acids (56). The truncated protein (Δ18) as well as the F918A/F922A mutant exhibited reduced activities. Our model does not implicate these residues in the direct binding with the HIV-1 IN CTD (supplemental Fig. S6). Instead, this C-terminal region establishes intra-protein contacts with the N-terminal part of TNPO3 to form a closed ring structure (Fig. 6A and supplemental Fig. S5). Accordingly, the C-ter-
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terminal truncation could significantly affect TNPO3 conformation. Furthermore, residues Phe-918 and Phe-922 are involved in the intra-helical interactions within HEAT 20

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REFERENCES

1. Hulme, A. E., Perez, O., and Hope, T. J. (2011) Complementary assays reveal a relationship between HIV-1 uncoating and reverse transcription. Proc. Natl. Acad. Sci. U.S.A. 108, 9975–9980

2. Brown, P. O. (1997) in Retroviruses (Coffin, J. M., Hughes, S. H., and Varnum, H. E., eds) pp. 161–204, Cold Spring Harbor Laboratory, Plainview, NY

3. Li, M., Mizuuchi, M., Burke, T. R., Jr., and Craigie, R. (2006) Retroviral DNA integration, reaction pathway, and critical intermediates. EMBO J. 25, 1295–1304

4. Miller, M. D., Farnet, C. M., and Bushman, F. D. (1997) Human immunodeficiency virus type 1 preintegration complexes, studies of organization and composition. J. Virol. 71, 5382–5390

5. Gallay, P., Hope, T., Chin, D., and Trono, D. (1997) HIV-1 infection of nondividing cells through the recognition of integrase by the importin/karyopherin pathway. Proc. Natl. Acad. Sci. U.S.A. 94, 9825–9830

6. Yamashita, M., and Emerman, M. (2004) Capsid is a dominant determinant of retrovirus infectivity in nondividing cells. J. Virol. 78, 5670–5678

7. von Schwedler, U., Kornbluth, R. S., and Trono, D. (1994) The nuclear localization signal of the matrix protein of human immunodeficiency virus type 1 allows the establishment of infection in macrophages and quiescent T lymphocytes. Proc. Natl. Acad. Sci. U.S.A. 91, 6992–6996

8. Gallay, P., Stitt, V., Mundy, C., Oettinger, M., and Trono, D. (1996) Role of the karyopherin pathway in human immunodeficiency virus type 1 nuclear import. J. Virol. 70, 1027–1032

9. Hearps, A. C., and Jans, D. A. (2006) HIV-1 integrase is capable of targeting DNA to the nucleus via an importin α/β-dependent mechanism. Biochem. J. 398, 475–484

10. Brass, A. L., Dykhooorn, D. M., Benita, Y., Yan, N., Engelman, A., Xavier, R. I., Lieberman, J., and Elledge, S. J. (2008) Identification of host proteins required for HIV infection through a functional genomic screen. Science 319, 921–926

11. Fassati, A., Görlöich, D., Harrison, I., Zaytseva, L., and Mintog, J. M. (2003) Nuclear import of HIV-1 intracellular reverse transcription complexes is mediated by importin-7. EMBO J. 22, 3675–3685

12. Christ, F., Thys, W., De Rijck, J., Gijsbers, R., Albanese, A., Arosio, D., Emiliani, S., Rain, J. C., Benarous, R., Cereseto, A., and Debyser, Z. (2008) Transportin-SR2 imports HIV into the nucleus. Curr. Biol. 18, 1192–1202

13. König, R., Zhou, Y., Eldered, D., Diamond, T. L., Bonamy, G. M., Irelan, J. T., Chiang, C. Y., Tu, B. P., De Jesus, P. D., Lilley, C. E., Seidel, S., Opaluch, A. M., Caldwell, J. S., Weitzman, M. D., Kuhnen, K. L., Bandypadhyay, S., Eldered, T., Orph, A. P., Miraglia, L. J., Bushman, F. D., Young, J. A., and Chanda, S. K. (2008) Global analysis of host-pathogen interactions that regulate early-stage HIV-1 replication. Cell 135, 49–60

14. Ociewia, K. E., Brady, T. L., Ronen, K., Hugel, A., Roth, S. L., Schaller, T., James, L. C., Towers, G. J., Young, J. A., Chanda, S. K., König, R., Malani, N., Berry, C. C., and Bushman, F. D. (2011) HIV integration targeting. A pathway involving transportin-3 and the nuclear pore complex RanBP2. PLoS Pathog. 7, e1002349

15. Matreyek, K. A., and Engelman, A. (2011) The requirement for nucleoporin NUP153 during human immunodeficiency virus type 1 infection is determined by the viral capsid. J. Virol. 85, 7818–7827

16. Schaller, T., Ociewia, K. E., Rasaiyaiah, J., Price, A. J., Brady, T. L., Roth, S. L., Hué, S., Fletcher, A. J., Lee, K., KewalRamani, V. N., Noursadeghi, M., Jenner, R. G., James, L. C., Bushman, F. D., and Towers, G. J. (2011) HIV-1 capsid-cyclophilin interactions determine nuclear import pathway, integration targeting, and replication efficiency. PLoS Pathog. 7, e1002439

17. Bushman, F. D., Malani, N., Fernandes, J., D’Orso, I., Cagny, G., Diamond, T. L., Zhou, H., Hazuda, D. J., Espeas, A. S., König, R., Bandypadhyay, S., Eldered, T., Goff, S. P., Krogan, N. J., Frank, A. D., Young, J. A., and Chanda, S. K. (2009) Host cell factors in HIV replication. Meta-analysis of genome-wide studies. PLoS Pathog. 5, e1000437

18. Kataoka, N., Bachorik, J. L., and Dreyfuss, G. (1999) Transportin-SR, a nuclear import receptor for SR proteins. J. Cell Biol. 145, 1145–1152

19. Krishnan, L., Matreyek, K. A., Oztop, I., Lee, K., Tipper, C. H., Li, X., Dar, M. J., KewalRamani, V. N., and Engelman, A. (2010) The requirement for cellular transportin 3 (TNPO3 or TRN-SR2) during infection maps to human immunodeficiency virus type 1 capsid and not integrase. J. Virol. 84, 397–406

20. Thys, W., De Houver, S., Demeulemeester, J., Taltuy, O., Vancaenbroeck, R., Gérard, M., De Rijck, J., Gijsbers, R., Christ, F., and Debyser, Z. (2011) Interplay between HIV entry and transportin-SR2 dependency. Retrovirology 8, 7

21. Lee, K., Ambrose, Z., Martin, T. D., Oztop, I., Mullky, A., Julias, J. G., Vandegraaff, N., Baumann, J. G., Wang, R., Yuen, W., Takamura, T., Shelton, K., Tanuiuchi, I., Li, Y., Sodrok, I., Litman, D. R., Coffin, J. M., Hughes, S. H., Untumat, D., Engelman, A., and KewalRamani, V. N. (2010) Flexible use of nuclear import pathways by HIV-1. Cell Host Microbe 7, 221–233

22. De Iaco, A., and Luban, J. (2011) Inhibition of HIV-1 infection by TNPO3 depletion is determined by capsid and detectable after viral cDNA enters the nucleus. Retrovirology 8, 98

23. Vallee-Casuso, J. C., Di Nunzio, F., Yang, Y., Resnka, N., Lienlauf, M., Arzel, N., Perez, P., Brass, A. L., and Diaz-Griffero, F. (2012) TNPO3 is required for HIV-1 replication after nuclear import but prior to integration and binds the HIV-1 core. J. Virol. 86, 5931–5936

24. Zhou, L., Sokolsska, J., Eolly, J., James, W., Cowley, S. A., and Fassati, A. (2011) Transportin 3 promotes a nuclear maturation step required for efficient HIV-1 integration. PLoS Pathog. 7, e1002194

25. Kessl, J. J., Jena, N., Koh, Y., Taskent-Sezgin, H., Slaughter, A., Feng, L., Shkriabai, N., Dar, M. J., Kvaratskhelia, M. (2008) Dynamic modulation of HIV-1 integrase structure and function and HIV-1 replication targeting, and replication efficiency. PLoS Pathog. 7, e1002194

26. Cherepanov, P. (2007) LEDGF/p75 interacts with divergent lentiviral integrase and modulates their enzymatic activity in vitro. Nucleic Acids Res. 35, 113–124

27. McKee, C. J., Kessl, J. J., Shkriabai, N., Dar, M. J., Engelman, A., and Kvaratskhelia, M. (2008) Dynamic modulation of HIV-1 integration structure and function by cellular lens epithelium-derived growth factor (LEDFG) protein. J. Biol. Chem. 283, 31802–31812
71. Lai, M. C., Lin, R. I., Huang, S. Y., Tsai, C. W., and Tarn, W. Y. (2000) A human importin-β family protein, transportin-SR2, interacts with the phosphorylated RS domain of SR proteins. *J. Biol. Chem.* **275**, 7950–7957

72. Jayappa, K. D., Ao, Z., Yang, M., Wang, J., and Yao, X. (2011) Identification of critical motifs within HIV-1 integrase required for importin α3 interaction and viral cDNA nuclear import. *J. Mol. Biol.* **410**, 847–862

73. Li, X., Koh, Y., and Engelman, A. (2012) Correlation of recombinant integrase activity and functional preintegration complex formation during acute infection by replication-defective integrase mutant human immunodeficiency virus. *J. Virol.* **86**, 8919–8925

74. Fassati, A., and Goff, S. P. (1999) Characterization of intracellular reverse transcription complexes of Moloney murine leukemia virus. *J. Virol.* **73**, 8919–8925

75. Fassati, A., and Goff, S. P. (2001) Characterization of intracellular reverse transcription complexes of human immunodeficiency virus type 1. *J. Virol.* **75**, 3626–3635

76. Görlich, D., Dabrowski, M., Bischoff, F. R., Kutay, U., Bork, P., Hartmann, E., Prehn, S., and Izaurralde, E. (1997) A novel class of RanGTP-binding proteins. *J. Cell Biol.* **138**, 65–80

77. Bhardwaj, A., and Cingolani, G. (2010) Conformational selection in the recognition of the snurportin importin β binding domain by importin β. *Biochemistry* **49**, 5042–5047

78. Cansizoglu, A. E., Lee, B. J., Zhang, Z. C., Fontoura, B. M., and Chook, Y. M. (2007) Structure-based design of a pathway-specific nuclear import inhibitor. *Nat. Struct. Mol. Biol.* **14**, 452–454

79. Cingolani, G., Petosa, C., Weis, K., and Müller, C. W. (1999) Structure of importin-β bound to the IBB domain of importin-α. *Nature* **399**, 221–229

80. Lee, B. J., Cansizoglu, A. E., Süel, K. E., Louis, T. H., Zhang, Z., and Chook, Y. M. (2006) Rules for nuclear localization sequence recognition by karyopherin B2. *Cell* **126**, 543–558

81. Lee, S. J., Sekimoto, T., Yamashita, E., Nagoshi, E., Nakagawa, A., Imamoto, N., Yoshimura, M., Sakai, H., Chong, K. T., Tsukihara, T., and Yoneda, Y. (2003) The structure of importin-β bound to SREBP-2. Nuclear import of a transcription factor. *Science* **302**, 1571–1575

82. Mitrousis, G., Olia, A. S., Walker-Kopp, N., and Cingolani, G. (2008) Molecular basis for the recognition of snurportin 1 by importin β. *J. Biol. Chem.* **283**, 7877–7884

83. Wohlwend, D., Strasser, A., Dickmanns, A., and Ficner, R. (2007) Structural basis for RanGTP independent entry of spliceosomal U snRNPs into the nucleus. *J. Mol. Biol.* **374**, 1129–1138