TNPO3-mediated nuclear entry of the Rous sarcoma virus Gag protein is independent of the cargo-binding domain

Breanna L. Rice[a], Matthew S. Stake[a*], and Leslie J. Parent[a,b,#]

[a]Division of Infectious Diseases and Epidemiology, Department of Medicine, Penn State College of Medicine, Hershey, PA, USA

[b]Department of Microbiology and Immunology, Penn State College of Medicine, Hershey, PA, USA

Running Head: TNPO3-mediated nuclear entry of alpharetrovirus Gag

#Address correspondence to Leslie Parent, lparent@psu.edu.

*Present address:

Matthew S. Stake

UPMC Hanover Medical Group, Hanover, PA, USA

B.L.R and M.S.S. contributed equally to this work.

Abstract

Retroviral Gag polyproteins orchestrate the assembly and release of nascent virus particles from the plasma membranes of infected cells. Although it was traditionally thought that Gag proteins trafficked directly from the cytosol to the plasma membrane, we discovered that the oncogenic avian alpharetrovirus Rous sarcoma virus (RSV) Gag protein undergoes transient nucleocytoplasmic transport as an intrinsic step in virus assembly. Using a genetic approach in yeast, we identified three karyopherins that engage the two independent nuclear localization signals (NLSs) in Gag. The primary NLS is in the nucleocapsid (NC) domain of Gag and binds directly to importin-α, which recruits importin-β to mediate nuclear entry. The second NLS, which resides in the matrix (MA) domain, is dependent on importin-11 and transportin-3 (TNPO3), known as MTR10p and Kap120p in yeast, although it is not clear whether these import factors are independent or additive. The functionality of importin α/β and importin-11 has been verified in avian cells, whereas the role of TNPO3 has not been studied. In this report, we demonstrate that TNPO3 mediates nuclear entry of Gag and directly binds to Gag. To our surprise, this interaction did not require the cargo-binding domain of TNPO3, which typically mediates nuclear entry for other binding partners of TNPO3 including SR-domain containing splicing factors and tRNAs that re-enter the nucleus. These results suggest that RSV hijacks the host nuclear import pathway using a unique mechanism, potentially allowing other cargo to bind TNPO3 simultaneously.

Importance

RSV Gag nuclear entry is facilitated using three distinct host import factors that interact with nuclear localization signals in the Gag MA and NC domains. Here we

show that the MA region is required for nuclear import of Gag through the TNPO3 pathway. Gag nuclear entry does not require the cargo binding domain of TNPO3. Understanding the molecular basis for TNPO3-mediated nuclear trafficking of the RSV Gag protein may lead to a deeper appreciation for whether different import factors play distinct roles in retrovirus replication.

Introduction

The retrovirus structural protein Gag is a multi-domain protein that is responsible for packaging the viral genome and directing the assembly and budding of virus particles from the plasma membrane of infected cells. The matrix or MA domain of Gag facilitates membrane targeting and binding. The capsid or CA domain is important for Gag protein-protein interactions, as well forming the virus particle capsid. Nucleocapsid or NC binds to the viral RNA genome (vRNA) for packaging and is involved in protein-protein interactions.

It has been found that the Gag proteins from various retroviruses undergo nuclear trafficking [reviewed in (47)]. The mechanisms of Gag nuclear trafficking are not completely understood. For Rous sarcoma virus (RSV), it is hypothesized that the reason for Gag nuclear trafficking is binding vRNA for packaging (13). The nuclear export of RSV Gag was first discovered to be dependent on the CRM1 nuclear export protein that interacts with a nuclear export signal (NES) mapped to the p10 domain (Figure 1A) (41, 43). The MA and NC domains were later found to be involved in the nuclear import of Gag through their NLSs. Studies utilizing *Saccharomyces cerevisiae* mutants deficient in members of the Importin-β protein superfamily found that the NC domain undergoes nuclear entry through the Kap60p/Kap95p (mammalian Importin-α/β)

pathway, while MA uses either Kap120p (mammalian importin 11) or Mtr10p (mammalian transportin SR/ transportin-3/ TNPO3) (5). The interactions between the NC domain and the importin-α/β (Impα/β) complex, as well as the MA domain and importin 11 (Imp11) were confirmed via affinity-tagged purifications (14). Binding of Gag to nucleic acid *in vitro* impairs binding of Imp11 and Impα while simultaneously promoting the interaction with the nuclear export protein CRM1. These findings implied a model suggesting directionality of Gag nuclear trafficking: upon binding nucleic acid, Gag is readied for nuclear export into the cytoplasm and rendered less likely to shuttle back through the nucleus, Gag travels to the plasma membrane where it engages in terminal steps of virus assembly and budding (14).

Transportin-SR, or TNPO3, is a member of the Importin-β family of karyopherins (Figure 1B) (24, 31). TNPO3 serves as a nuclear import receptor for a class of evolutionarily conserved, essential splicing factors and pre-mRNA processing proteins known as serine and arginine-rich (SR) proteins. SR proteins derive their names from the serine/arginine enriched motifs in their C-terminal regions, and they also contain RNA recognition domains located at their N termini (45, 52). The RS domain of splicing factors interact directly with the C-terminal CBD of TNPO3, mediating nuclear entry of the splicing factor (17, 23). TNPO3 also mediates nuclear import of the pre-mRNA splicing factor RBM4 through its interaction with stretches of alanine residues, termed polyalanine domains (22). Viewed under steady state conditions, SR proteins and other spliceosomal components reside in discrete nuclear foci termed “speckles” (reviewed in (46)). TNPO3 and splicing factor cargoes localize to these structures using *in vitro*

nuclear import assay conditions, implicating TNPO3 in the intranuclear transport of SR proteins to speckles (22, 24, 25).

Additionally, TNPO3 has been shown to be important for several retroviruses. Small interfering RNA (siRNA) knockdowns of TNPO3 demonstrated that reduced TNPO3 expression impairs the infectivity of human immunodeficiency virus type 1 (HIV-1) (4, 7, 19). TNPO3 is involved in early events of HIV infection, primarily at the level of pre-integration complex (PIC) nuclear entry. *In vitro* and *in* vivo studies have shown direct interactions between TNPO3 and HIV integrase (9, 26, 29, 32, 49). In cells depleted of TNPO3 by small hairpin RNA (shRNA), HIV-1 PIC nuclear entry is impaired, as measured by a reduction in 2 long terminal repeat (LTR) circle formation, and integration is vastly reduced (11, 44, 50). Further, the cargo-binding domain of TNPO3 is required for rescuing HIV infection in cells depleted of TNPO3 by shRNA (31). Proviruses that do manage to integrate in TNPO3 knockdown cells do so in regions with lower gene density than the integration sites in control cells (37, 40).

There is also evidence suggesting that HIV CA governs TNPO3 sensitivity. Amino acid substitutions in CA, such as the N74D mutation, permit PIC nuclear entry in TNPO3 knockdown cells through an altered import pathway (10, 28). The actual mechanism by which TNPO3 depletion impairs HIV infectivity is not well understood but is believed to involve the cleavage and polyadenylation specificity factor subunit 6 (CPSF6), an SR protein. It is believed that the cytoplasmic portion of CPSF6 interacts directly with the HIV PIC to prevent nuclear import and/or alters PIC stability to disrupt the uncoating process (3, 11, 12, 38). A truncated form of CPSF6 was demonstrated to be involved in the rapid disassembly of the viral capsid and inhibition of cDNA synthesis

(16). A CA mutant bearing the N74D mutation binds CPSF6 *in vitro* with lower affinity than the wild-type CA, further implicating CPSF6 in PIC nuclear import (38). Some studies imply that TNPO3 is directly involved in the PIC uncoating process (44, 53).

Other studies have demonstrated that TNPO3 is important for the nuclear import of the foamy virus (FV) PIC as well. When TNPO3 expression is decreased in cells, the concentration of FV integrase (IN) in the nucleus decreased as well, but the nuclear localization of FV Gag was not affected demonstrating that FV IN utilizes TNPO3 for nuclear import (2). TNPO3 has also been shown to be important for the nuclear import of the virus during infection of simian immunodeficiency virus mac239 and equine infectious anemia virus (21, 31), and the replication of human immunodeficiency virus type 2 (7) and bovine immunodeficiency virus (21). Not all retroviruses recruit TNPO3 for nuclear import; murine leukemia virus (MLV) replication is unaffected by TNPO3 knockdown (7, 21, 31) and feline immunodeficiency virus (FIV) replication seems to be weakly affected by TNPO3 knockdown (21, 31). *In vitro* affinity-tagged purifications were performed to examine TNPO3 interactions with various retroviral integrase proteins and it was found that while there was binding between MLV, FIV, and RSV integrase proteins with TNPO3, when TNPO3 expression is decreased in cells there does not appear to be an effect on viral replication for these three viruses (21). While it appears that TNPO3 is important for a number of retroviruses during early infection, the interaction of RSV Gag with TNPO3 during assembly has yet to be investigated in depth and is the subject of this work.

Results

Initial studies examining possible RSV Gag nuclear trafficking proteins discovered three import factors: Impβ, Imp11, and TNPO3 (5). Later it was confirmed that Imp11 and Impβ/Impα import Gag through interactions with MA and NC domains respectively. The interaction of RSV Gag with TNPO3 was not further explored until this time (14).

RSV Gag is a multi-domain protein as shown in Figure 1A. TNPO3 consists of three domains: the RanGTP binding domain (RBD), the nuclear pore complex (NPC) interaction domain, and the cargo binding domain (CBD) (Fig. 1A).

Gag.GFP is normally distributed in the nucleus, cytoplasm, and along the plasma membrane of avian cells in culture [Fig. 1B; (13, 18, 35, 41-43)]. Expression of mCherry.TNPO3 resulted in an increase in the amount of Gag protein within the nucleus (Fig. 1C, D). Quantitation of the amount of nuclear Gag fluorescence demonstrated an increase from 18.5% to 25% with exogenous TNPO3 expression (p-value < 0.0001) (Fig. 1D). Based on the finding that RSV MA nuclear entry depends on TNPO3/ Mtr10p in yeast (5), we wanted to test whether this interaction was functionally relevant in avian cells. To this end, two mutants were utilized that contain deletions in MA (Fig. 1A). One deletion mutant is missing the N-terminal portion of MA containing the previously-identified NLS (Gag.ΔMA_{5-86}), and the other has a large internal deletion of MA from residues 5-148 (Gag.ΔMA_{5-148}). Nuclear localization of both MA mutants, Gag.ΔMA_{5-86} and Gag.ΔMA_{5-148}, was slightly reduced compared to full-length Gag, with quantitative analysis showing 15.5% and 12% of mutant Gag was nuclear, respectively (Fig. 1B, D). When exogenous TNPO3 was expressed, the nuclear Gag percentages became 16.5% and 13% for Gag.ΔMA_{5-86} and Gag.ΔMA_{5-148}, respectively, demonstrating no significant

increase in Gag nuclear localization (Fig. 1C, D). These results suggest that the activity of TNPO3 in mediating nuclear entry of Gag is dependent on the MA domain. By contrast, when the NC domain of Gag was deleted (Gag.ΔNC, Fig. 1A), a result similar to wild-type Gag was observed, with a baseline of 17% nuclear localization for Gag.ΔNC, increasing to 22% ($p < 0.0001$; Fig. 1B-D) with exogenous TNPO3 expression. This result was expected due to previous results indicating that nuclear import of RSV NC was dependent on the importin α/β complex (5, 14) but not TNPO3, validating our previous findings using a genetic screen in *S. cerevisiae*.

The next goal was to identify which domain of TNPO3 was required for Gag nuclear import in avian cells. We expected that the cargo-binding domain (CBD) would be necessary because of previous findings demonstrating SR- splicing factors bind directly to the CBD for nuclear import (17, 23). To test this hypothesis, a TNPO3.ΔCargo mutant was co-expressed in cells with Gag.YFP (Fig. 2A). Unexpectedly, there was an increase in the amount of nuclear Gag from 19% to 26% ($p < 0.0001$) (Fig. 2B, C).

The interaction between Gag and TNPO3 was further examined through *in vitro* affinity-tagged purification to identify a direct protein-protein interaction. Wild-type Gag, GST-tagged TNPO3, and their respective deletion mutants (Fig. 3A) were expressed in *E. coli* and purified. RSV Gag was incubated with GST-TNPO3, and the protein complexes were affinity purified using GST beads and separated by SDS-PAGE. Full-length Gag protein was strongly associated with GST-TNPO3 and not with the GST protein tag (Fig. 3B). To determine whether the Gag-TNPO3 interaction was dependent on the presence of the membrane-binding MA domain, a Gag mutant containing a

deletion of the first 82 amino acids of MA (ΔMBD.Gag) were tested (5, 41). ΔMBD.Gag showed reduced binding to GST-TNPO3 compared to full-length Gag, demonstrating that the first 82 amino acids of Gag are important for the interaction with TNPO3 (Fig. 3B). Additional Gag mutants were used to further define Gag-TNPO3 interaction sites, including a truncation at the end of the CA domain (Gag.ΔSPΔNC), a deletion of the C-terminal domain of CA through the end of NC (MA.p2.p10.CA-NTD), a mutant expressing only CA and NC (CA.NC), and a MA-only expression construct. It was found that each Gag mutant, and surprisingly the MA deletion mutants ΔMBD and CA.NC, associated with GST-TNPO3, albeit to a much lesser extent than full-length Gag (Fig. 3B, C).

Next, we examined which domain of TNPO3 was required for binding to Gag. We utilized the mutant that lacked the CBD (GST-TNPO3.ΔCargo) as well as a mutant that contains only the NPC binding domain in the central region of TNPO3 (GST-TNPO3.NPC) (Fig. 3A). We incubated TNPO3.ΔCargo or TNPO3.NPC with full-length Gag, and both proteins bound very strongly to full-length Gag (Fig. 3B, C). By contrast, there were only very weak interactions between TNPO3.ΔCargo and each of the Gag truncation mutants, including ΔMBD.Gag, CA.NC, Gag.ΔSPΔNC, and MA.p2.p10.CA-NTD (Fig. 3B). When MA was incubated with full-length TNPO3 or TNPO3 deletion mutants, MA showed the strongest binding to full-length TNPO3, reduced binding toTNPO3.NPC, and very little, if any, binding of MA with TNPO3.ΔCargo. This is demonstrated through the 120s exposure of MA, where with full-length TNPO3, the MA band is over-saturated, in order to see any interaction with TNPO3.ΔCargo (Fig. 3B,C). These results demonstrate that the requirement for binding of Gag and TNPO3 required

a full-length Gag protein with a TNPO3 construct that contains, at a minimum, the NPC binding domain of TNPO3.

To better understand the relationship between TNPO3 and the other host factors involved in Gag nuclear import, the TNPO3 overexpression experiments were expanded to include other karyopherins identified to interact with Gag MA and NC NLSs. Overexpression of two import factors that bind distinct Gag NLSs were predicted to cooperate and import more Gag into the nucleus. Conversely, overexpression of two import factors that bind the same NLS may compete for binding sites on Gag, resulting in no further increase in nuclear entry. When each individual import factor was co-expressed with Gag, the percentage of Gag in the nucleus significantly increased. Co-expression of TNPO3 resulted in an increase 15% nuclear Gag to 21%, Imp11 co-expression increased nuclear Gag from 15% to 21%, and Impβ co-expression increased nuclear Gag from 15% to 25% (each $p < 0.0001$) (Fig. 4A, C). When TNPO3 and Imp11 were co-expressed in cells, the amount of nuclear Gag increased to 21%, which was not significantly different compared to TNPO3 or Imp11 expression alone. However, when TNPO3 and Impβ were both overexpressed, there was an increase in nuclear Gag from 21% (TMPO3 alone) to 28% (TNPO3 plus Impβ; $p < 0.0001$) (Fig. 4B, C). These data demonstrate that Impβ and TNPO3 import Gag in an additive fashion, suggesting they have different binding sites in Gag. By contrast, TNPO3 and Imp11 may compete for the same NLS in the MA region of Gag and therefore are unable to increase the amount of Gag nuclear import when co-expressed.

Discussion

Nuclear trafficking of RSV Gag is important for efficient encapsidation of the viral genome (13, 41). To accomplish this vital step in virus replication, Gag uses multiple NLSs and host import pathways to gain entry into the nucleus. Previous studies revealed that Impα/β binds directly to a classical NLS in the NC domain of RSVGag whereas Imp11 interacts directly with a noncanonical NLS in the MA domain of RSV Gag (14). Although we had identified TNPO3 (Mtr10p) as important for entry of MA in yeast (5), we wondered whether this finding would be verified in higher eukaryotic cells given that the MA sequence does not contain an RS-rich motif or a polyalanine domain characteristic of known TNPO3 cargoes (21, 22). Here it has been shown both *in vitro* and *in vivo* that there is indeed an interaction between TNPO3 and RSV Gag that appears to be mediated through the MA domain and it is functional in the nuclear import of RSV Gag.

We also demonstrated that overexpression of TNPO3 resulted in increased accumulation of Gag in the nucleus (Fig. 1) in an MA-dependent fashion. However, there was a small amount of the MA deletion mutant that was pulled down by GST-TNPO3 (Fig. 3), indicating that there are other regions of Gag that contribute to the interaction with TNPO3, albeit to a much lesser extent than MA. In cells, the normal function of TNPO3 is to import splicing factors into the nucleus through direct interactions between the C-terminal cargo-binding domain (CBD) and the RS or polyalanine domain of splicing factor cargoes (22, 23). We anticipated that Gag would bind the cargo domain of TNPO3, being a potential cargo itself; however, we found that the CBD was dispensable (TNPO3.ΔCargo) for the interaction with Gag. Further supporting this observation, TNPO3.ΔCargo expression in cells led to a significant

increase in nuclear localization of Gag. Surprisingly, Gag bound efficiently to the purified NPC binding domain of TNPO3 (Fig. 3). Taken together, these results suggest that the major points of contact are the Gag MA region and the TNPO3 NPC binding domain, although it is important to note that other regions of Gag and TNPO3 appear to be required for maximal binding to occur. A recent study demonstrated that the N-terminus of TNPO3 (containing the RanGTP binding domain and part of the NPC interaction domain) interacts with the catalytic core domain and C-terminal domain of HIV-1 integrase, suggesting that other retroviral cargoes of TNPO3 bind to sites outside of the CBD (49).

The experiments presented up to this point and all previous studies examining RSV Gag nuclear import examined only one import factor at a time (5, 14). Therefore, cooperativity between factors responsible for Gag nuclear import was examined as a means to understand the relationship among host factors implicated in Gag nuclear import. Data presented in Fig. 4 indicates that TNPO3 can cooperate with Impβ but not Imp11 to drive Gag into the nucleus. This finding implies that factors interacting with different domains of Gag (i.e., TNPO3 with MA and Impα/Impβ with NC) are able to function in an additive manner to mediate Gag nuclear import. These findings also suggest that factors binding to the same domain of Gag (i.e. TNPO3 and Imp11 both bind to MA) cannot cooperate for Gag nuclear import, potentially because they bind to the same site or to sites in close proximity to one another.

The pleiotropic nature of Gag makes it extremely difficult to introduce mutations that affect only one aspect of Gag function. For instance, the same region of MA that contains the NLS also functions as the plasma membrane binding and targeting signal

for Gag during virion assembly (51). In a similar manner, the residues comprising the NC NLS are also required for genomic RNA packaging (5, 27). Given the multiple functions of these domains, introducing point mutations to abolish the binding of single karyopherins may not be practical, as it would be difficult to distinguish phenotypes resulting from an importin binding defect from disruption in subcellular trafficking of Gag. There is also a drawback to altering levels of endogenous import factors in cells, because the lack of a definable phenotype in such cells could be attributed to functional redundancy of the remaining import factors.

One question that remains is why RSV Gag utilizes three different nuclear import pathways. One possibility is that each import pathway is used to reach a particular location in the nucleus. For example, Impβ transports a variety of proteins into the nucleus including transcription factors, cell cycle regulators, histones, and ribosomal proteins [reviewed in (6)]. Imp11 transports ribosomal proteins and E2-ubiquitin-conjugating enzymes [reviewed in (6)]. TNPO3 transports SR splicing factors to splicing speckles (22-24), which are adjacent to transcription sites (46). Splicing factors are activated through phosphorylation, triggering them to traffic to sites of active transcription to process newly transcribing RNAs (34, 36), with rapid exchange of splicing factors between sites of transcription and speckles (20, 48). We previously showed that a nuclear-localized mutant of RSV Gag (Gag.L219A) has a high degree of colocalization with the phosphorylated forms of SR protein splicing factors SF2 and SC35 (39). Thus, it is possible that Gag enters the nucleus using the TNPO3 pathway to reach nuclear speckles adjacent to sites of nascent mRNA transcription (17, 48).

Further experiments will need to be performed to dissect the precise role of TNPO3 in Gag nuclear trafficking and RSV biology.

Materials and Methods

Cells and Plasmids

QT6 quail fibroblast cells were maintained as described in (8) and were transfected with the calcium phosphate precipitation method.

Tissue culture expression: Gag.GFP was described in (41). Gag.ΔMA$_{5-86}$.GFP and Gag.ΔMA$_{5-148}$.GFP were described previously (5). Gag.YFP and Gag.ΔNC.YFP were previously described (18). GFP-TNPO3 and GFP-TNPO3.ΔCargo (31) were gifts of Nathaniel Landau (NYU Langone Medical Center). Ceruleun.TNPO3.CBD was a generous gift from Yaron Shav-Tal (Bar Ilan University). mCherry.TNPO3 was created by PCR amplifying the TNPO3 coding sequence from GFP.TNPO3 with flanking *Xho*I and *Sal*I restriction sites and inserting into mCherry.N2 digested with those same enzymes. pKH3.Importin 11 and pKH3.Importin β, encoding HA.Importin 11 and HA.Importin β, respectively, were described in (14).

E. coli protein expression: pET28.TEV-Gag.3h encoding RSV Gag with an N-terminal cleavable 6-histidine tag (Gag) is described in (14). Gag.ΔNC.ΔSP was created by inserting two consecutive, in-frame stop codons into pET28.TEV.Gag.3h preceding the SP coding sequence. Gag.ΔMBD was created by PCR amplification of the Gag coding region starting at amino acid 83 and terminating at the N-terminus of NC with the insertion of stop codons. This product was ligated into appropriately digested pET24a+. CA.NC was created by PCR amplification of CA and NC inserting a NdeI site at the N-terminus and a HindIII site at the C-terminus. The amplicon was inserted into the

digested pET28(-His).Gag.ΔPR using the same sites. MA.p2.p10.CA-NTD was created similarly as CA.NC with the NdeI site at the N-terminus of MA and two STOP codons followed by the HindIII site after the 444 nt of CA. MA was described previously (14). pGEX6P3.hTNPO3 (21) encoding GST.TNPO3 for bacterial expression was a gift from Alan Engelman (Dana Farber Cancer Institute). GST.TNPO3.ΔCargo$_{1\text{-}501}$ was created from pGEX6P3.hTNPO3 by inserting three in frame, consecutive stop codons following the sequence encoding amino acid 501 of TNPO3 by Quikchange PCR mutagenesis (30). GST.TNPO3.NPC was created by deleting most of the Ran binding domain using primers: 5' – GGA GAA AAC CTT TAC TTC CAG GG-3' and 5' – AAT GGA TCC CAG GGG CCC-3' using the Q5 Site-Directed Mutagenesis protocol according to the manufacturer guidelines (New England Biolabs). Michael Malim (King's College London) kindly provided the bacterial expression construct encoding GST-Importin β.

Cell fixation and immunofluorescence

QT6 cells were grown on glass coverslips and fixed with 2% paraformaldehyde (PFA) in phosphate buffered saline (PBS) (supplemented with 5 mM EGTA and 4 mM $MgCl_2$, and adjusted to pH 7.2-7.4 with HCl) or 3.7% PFA in 2x PHEM buffer (3.6% PIPES, 1.3% HEPES, 0.76%EGTA, 0.198% MgSO4, pH to 7.0 with 10M KOH) (33) for 15 minutes at room temperature. Cells expressing HA.Importin β or 11 were then permeabilized with ice cold 100% methanol for 2 minutes on ice and blocked in 5% goat serum (Rockland) diluted into PBS for at least 2 hours at room temperature. Cells were stained with mouse anti-HA antibody (Genscript) diluted 1:500 in PBS supplemented with 0.5% goat serum and 0.01% Tween-20 (Sigma) for at least one hour in a humidified chamber.

After primary antibody incubation, the cells were incubated with goat anti-mouse antibody conjugated to Cy5 (Molecular Probes) diluted 1:500 or Alexa 647 (Life Technologies) diluted 1:1000 in PBS for 1 hour at room temperature. Cells were stained with DAPI at 5 µg/ml and mounted with Slow-Fade mounting medium (Molecular Probes) or Prolong Diamond (Thermo Fisher Scientific).

Microscopy

Images were captured either using a DeltaVision DV Elite (Applied Precision) wide field deconvolution microscope with a 60x oil immersion objective or a Leica SP8 TCS scanning confocal microscope equipped with a 63X oil immersion objective and White Light Laser (WLL). For the DeltaVision, 0.2 µm optical slices encompassing the entire cell were captured and deconvolved with softWoRx 5.0 (Applied Precision). From the deconvolved image stack, a single slice encompassing the widest section of the nucleus was exported for each channel as an uncompressed TIFF file for subsequent display and analysis. For images acquired using the SP8, we employed sequential scanning between frames, averaging four frames per image. The 405 nm UV laser was used to image the DAPI signal 10% laser power with an emission detection window of 415 – 466 nm using the PMT detector. Cerulean was imaged using the Argon 458 nm laser and a hybrid detector window of 463 – 499 nm. GFP was imaged using the WLL excited with the 489 nm laser line and a hybrid detector window of 495 – 559 nm. YFP was imaged using the WLL with a laser line excitation of 514 nm and a hybrid detector window of 519 – 569 nm. mCherry was imaged using the WLL with a laser line excitation of 587 nm and a hybrid detector window of 592 – 656 nm. All channels using the hybrid detectors had a time gating of 0.3 to 6.0 ns.

Quantitation of nuclear localization

ImageJ software version 1.46m was used to analyze cells for the amount of Gag present in the nucleus, as determined by fluorescence signal (1). The sum of all the pixel intensities in the Gag channel, expressed in ImageJ as the “Integrated Density,” for a region encompassing the entire cell was divided by the integrated density of the nucleus to calculate the percentage of the total cellular Gag pool residing in the nucleus. Outliers with a *p* value less than 0.05 as determined by Grubbs’ test (GraphPad Software Inc, <http://graphpad.com/quickcalcs/Grubbs1.cfm>) were removed from subsequent analyses. GraphPad Prism 5 (GraphPad Software, Inc.) was used to create all graphs, perform linear regression analysis, calculate the mean, and determine *p* values. *p* values were calculated by unpaired *t*-test as only pairs (i.e. no more than two) of conditions are analyzed simultaneously.

Expression and purification of recombinant RSV Gag proteins

All constructs for protein expression and purification were transformed into BL21 Gold DE3 pRIL *E. coli*. The purity of all protein preps was verified with Coomassie staining following SDS-PAGE and/or western blot analysis with appropriate antibodies. All sonications were performed on ice with an S-4000 sonicator (Misonix, Inc.) using a ½” tip. All Gag constructs were expressed in ZYP-5052 autoinduction medium, lysed in BugBuster Primary Amine Free Protein Extraction Reagent (Novagen) supplemented with recombinant lysozyme. PEI was added to a final concentration of 0.15% and lysate was centrifuged at 21,000 RCF for 30 minute to remove cell debris. The protein remaining in the soluble fraction was precipitated for 30 minute at room temperature with concentrated ammonium sulfate. The pellet containing the Gag protein was

resuspended and clarified by centrifugation prior to chromatographic separation and elution with a sulfopropyl cation exchange column. Peak eluted fractions were dialyzed against 25 mM HEPES pH 7.5, 500 mM NaCl, 0.1 mM EDTA, 0.1 mM TCEP, and 0.01 mM $ZnSO_4$ prior to concentration, aliquoting, and storage at -80ºC.

Expression and purification of recombinant GST-tagged proteins

Purified GST protein was a gift from John Flanagan (Penn State College of Medicine). GST.TNPO3 was grown in two 250 ml cultures of ZYP-5052 supplemented with ampicillin and incubated at 37ºC for 19.5 hours. Cell pellets were harvested by centrifugation and stored at -20ºC prior to purification. A batch purification protocol adapted from (15) was used to purify GST.TNPO3. Cell pellets were thawed on ice, homogenized into PBS containing Roche Complete EDTA free protease inhibitors (Roche). Ready-lyse lysozyme and Omnicleave nuclease (Epicentre) were added and the mixture was permitted to rock on ice for 15 minutes. The homogenate was then sonicated three times at 80% power, with a one-minute recovery between each sonication. Lysate was clarified for 30 min at 21,000 RCF at 4ºC and passed through a 0.45 µm filter. The soluble portion was incubated with gentle end over end mixing for 3 hours at 4ºC with Glutathione Sepharose 4 Fast Flow Beads (GE) prewashed with PBS. Following binding, the beads were washed three times with PBS to remove unbound proteins. Bound proteins were eluted with a 30 minute incubation at 4ºC with Elution Buffer (50 mM Tris pH 8.0, 40 mM reduced glutathione, and Roche Complete protease inhibitors). At the end of the incubation, beads were pelleted and the supernatant removed to a prechilled tube. Two additional elution steps were performed. All elutions were pooled and dialyzed against TNPO3 storage buffer (50 mM HEPES pH 7.4, 150

mM NaCl, 10% Glycerol, and 2 mM DTT). Following dialysis, purified GST.TNPO3 was concentrated with an Amicon Ultra Centrifugal Filter Device (Millipore), aliquoted, and stored at -80ºC prior to use. GST.TNPO3.ΔCargo and GST.TNPO3.NPC were expressed at 30ºC, but otherwise expressed and purified identically to GST.TNPO3.

In vitro GST affinity purification protein-protein interactions assays

The protocol for GST affinity purification assays was adapted from (21). All proteins used in purification assays were performed at equimolar of 185 nM in 540 µl pull-down buffer (150 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, 0.1% NP-40, 25 mM Tris-Cl pH 7.4). For the input gel, 40 µl was removed. Proteins were incubated for 1 hour at room temperature with gentle end over end rotation. 60 µl of a 50% slurry of glutathione beads (Glutathione Sepharose 4 Fast Flow, GE) prewashed four times in pull-down buffer were then added to the complexes and incubated for 2 hours at room temperature with gentle end over end rotation. The beads were pelleted by centrifugation at 800x*g* for 2 minutes. The supernatant containing unbound proteins was removed and the beads containing the bound protein complexes were washed four times with 10 packed bead volumes of pull down buffer. Following the final wash, one packed bead volume of elution buffer (25 mM Tris-Cl and 40 mM reduced glutathione, pH 8) was added, mixed with the beads, and placed on ice for 10 minutes to elute the bound complexes. Following elution, beads were pelleted by centrifugation at 800x*g* for 2 minutes at 4°C. The supernatant containing bound complexes was then removed to a clean microcentrifuge tube and 4x SDS-PAGE loading buffer (250 mM Tris-HCl, pH 6.8, 40% glycerol, 0.4% bromophenol blue, 8% SDS, and 8% β-mercaptoethanol) was added to 1x final concentration. The samples were then heated for 5 minutes at 85ºC

and separated by SDS-PAGE and analyzed by Western blot using rabbit anti-GST antibody (Genscript), rabbit α-RSV CA, rabbit α-RSV MA.p2, rabbit α-RSV MA.MDB, and HRP-conjugated secondary antibodies (Invitrogen). RSV antibodies were generous gifts from Rebecca Craven (Penn State College of Medicine). The input gels were visualized with Acquastain dye (Bulldog Bio).

Acknowledgments

We would like to thank the following scientists for their generosity in supplying reagents, Dr. Nathaniel Landan (NYU Langone Medical Center), Dr. Alan Engelman (Dana Farber Cancer Institute), Dr. Michael Malim (King's College London), Dr. Yaron Shav-Tal (Bar Ilan University), Dr. John Flanagan and Dr. Rebecca Craven (PSU College of Medicine). Special thanks to Malgorzata Sudol in the Department of Medicine at the Penn State College of Medicine for technical assistance. We would like to acknowledge the Microscopy Imaging Core Facility at PSU College of Medicine for use of the confocal [Leica SP8- 1S10OD010756-01A1 (CB)] and deconvolution microscopes and the Imaris (Bitplane imaging analysis software. This project was funded in part by NIH R01 CA076534 (LJP) and F31 CA196292 (BLR).

References

1. **Abramoff, M. D., P. J. Magalhaes, and S. J. Ram.** 2004. Image Processing with ImageJ. Biophotonics International **11:**36-42.
2. **Ali, M. K., J. Kim, F. B. Hamid, and C.-G. Shin.** 2015. Knockdown of the host cellular protein transportin 3 attenuates prototype foamy virus infection. Bioscience, Biotechnology, and Biochemistry **79:**943-951.
3. **Bhattacharya, A., S. L. Alam, T. Fricke, K. Zadrozny, J. Sedzicki, A. B. Taylor, B. Demeler, O. Pornillos, B. K. Ganser-Pornillos, F. Diaz-Griffero, D. N. Ivanov, and M. Yeager.** 2014. Structural basis of HIV-1 capsid recognition by PF74 and CPSF6. Proceedings of the National Academy of Sciences **111:**18625-18630.

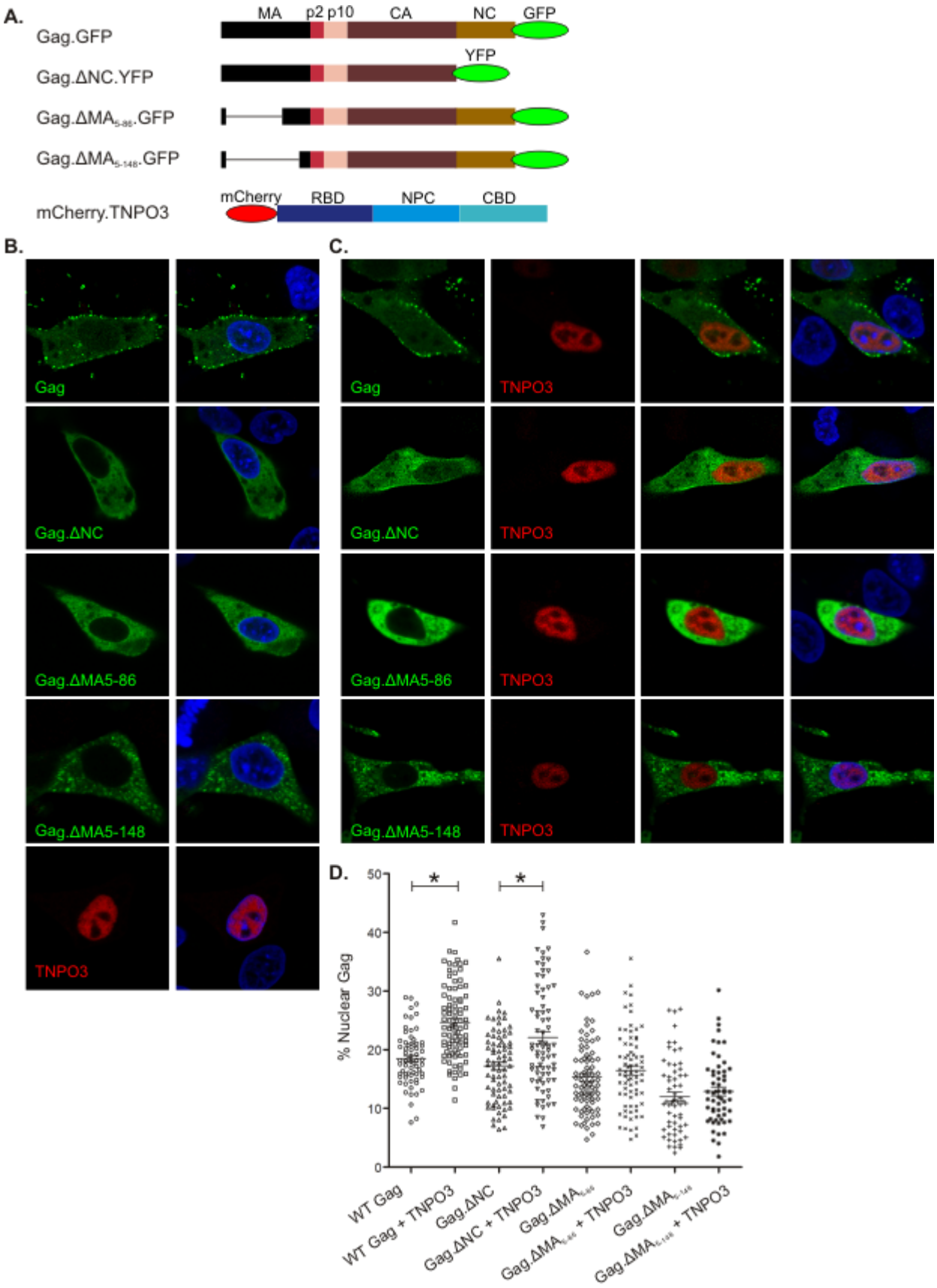
4. **Brass, A. L., D. M. Dykxhoorn, Y. Benita, N. Yan, A. Engelman, R. J. Xavier, J. Lieberman, and S. J. Elledge.** 2008. Identification of Host Proteins Required for HIV Infection Through a Functional Genomic Screen. Science **319:**921-926.
5. **Butterfield-Gerson, K. L., L. Z. Scheifele, E. P. Ryan, A. K. Hopper, and L. J. Parent.** 2006. Importin-β Family Members Mediate Alpharetrovirus Gag Nuclear Entry via Interactions with Matrix and Nucleocapsid. J Virol **80:**1798-1806.
6. **Chook, Y. M., and K. E. Süel.** 2011. Nuclear import by karyopherin-βs: Recognition and inhibition. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research **1813:**1593-1606.
7. **Christ, F., W. Thys, J. De Rijck, R. Gijsbers, A. Albanese, D. Arosio, S. Emiliani, J. C. Rain, R. Benarous, A. Cereseto, and Z. Debyser.** 2008. Transportin-SR2 imports HIV into the nucleus. Curr Biol **18:**1192-1202.
8. **Craven, R. C., A. E. Leure-duPree, R. A. Weldon JR., and J. W. Wills.** 1995. Genetic Analysis of the Major Homology Region of the Rous Sarcoma Virus Gag Protein. Journal of Virology **69:**4213-4227.
9. **De Houwer, S., J. Demeulemeester, W. Thys, O. Taltynov, K. Zmajkovicova, F. Christ, and Z. Debyser.** 2012. Identification of Residues in the C-terminal Domain of HIV-1 Integrase That Mediate Binding to the Transportin-SR2 Protein. Journal of Biological Chemistry **287:**34059-34068.
10. **De Iaco, A., and J. Luban.** 2011. Inhibition of HIV-1 infection by TNPO3 depletion is determined by capsid and detectable after viral cDNA enters the nucleus. Retrovirology **8:**98.
11. **De Iaco, A., F. Santoni, A. Vannier, M. Guipponi, S. Antonarakis, and J. Luban.** 2013. TNPO3 protects HIV-1 replication from CPSF6-mediated capsid stabilization in the host cell cytoplasm. Retrovirology **10:**20.
12. **Fricke, T., J. C. Valle-Casuso, T. E. White, A. Brandariz-Nuñez, W. J. Bosche, N. Reszka, R. Gorelick, and F. Diaz-Griffero.** 2013. The ability of TNPO3-depleted cells to inhibit HIV-1 infection requires CPSF6. Retrovirology **10:**46-46.
13. **Garbitt-Hirst, R., S. P. Kenney, and L. J. Parent.** 2009. Genetic evidence for a connection between Rous sarcoma virus gag nuclear trafficking and genomic RNA packaging. J.Virol. **83:**6790-6797.
14. **Gudleski, N., J. M. Flanagan, E. P. Ryan, M. C. Bewly, and L. J. Parent.** 2010. Directionality of nucleocytoplasmic transport of the retroviral gag protein depends on sequential binding of karyopherins and viral RNA. Proceedings of the National Academy of Sciences **107:**9358-9363.
15. **Harper, S., and D. W. Speicher.** 2002. Expression and Purification of GST Fusion Proteins, vol. 1. John Wiley & Sons, Inc.
16. **Hori, T., H. Takeuchi, H. Saito, R. Sakuma, Y. Inagaki, and S. Yamaoka.** 2013. A Carboxy-Terminally Truncated Human CPSF6 Lacking Residues Encoded by Exon 6 Inhibits HIV-1 cDNA Synthesis and Promotes Capsid Disassembly. J Virol **87:**7726-7736.
17. **Kataoka, N., J. L. Bachorik, and G. Dreyfuss.** 1999. Transportin-SR, a Nuclear Import Receptor for SR Proteins. The Journal of Cell Biology **145:**1145-1152.

18. **Kenney, S. P., T. L. Lochmann, C. L. Schmid, and L. J. Parent.** 2008. Intermolecular Interactions between Retroviral Gag Proteins in the Nucleus. Journal of Virology **82:**683-691.
19. **König, R., Y. Zhou, D. Elleder, T. L. Diamond, G. M. C. Bonamy, J. T. Irelan, C.-y. Chiang, B. P. Tu, P. D. De Jesus, C. E. Lilley, S. Seidel, A. M. Opaluch, J. S. Caldwell, M. D. Weitzman, K. L. Kuhen, S. Bandyopadhyay, T. Ideker, A. P. Orth, L. J. Miraglia, F. D. Bushman, J. A. Young, and S. K. Chanda.** 2008. Global Analysis of Host-Pathogen Interactions that Regulate Early-Stage HIV-1 Replication. Cell **135:**49-60.
20. **Kornblihtt, A. R., M. de la Mata, J. P. Fededa, M. J. Munoz, and G. Nogues.** 2004. Multiple links between transcription and splicing. Rna **10:**1489-1498.
21. **Krishnan, L., K. A. Matreyek, I. Oztop, K. Lee, C. H. Tipper, X. Li, M. J. Dar, V. N. KewalRamani, and A. Engelman.** 2009. The Requirement for Cellular Transportin 3 (TNPO3 or TRN-SR2) during Infection Maps to Human Immunodeficiency Virus Type 1 Capsid and Not Integrase. Journal of Virology **84:**397-406.
22. **Lai, M.-C., H.-W. Kuo, W.-C. Chang, and W.-Y. Tarn.** 2003. A novel splicing regualtor shares a nuclear import pathway with SR proteins. The EMBO Journal **22:**1359-1369.
23. **Lai, M.-C., R.-I. Lin, S.-Y. Huang, C.-W. Tsai, and W.-Y. Tarn.** 2000. A Human Importin-ß Family Protein, Transportin-SR2, Interacts with the Phosphorylated RS Domain of SR Proteins. Journal of Biological Chemistry **275:**7950-7957.
24. **Lai, M. C., R. I. Lin, and W. Y. Tarn.** 2001. Transportin-SR2 mediates nuclear import of phosphorylated SR proteins. Proceedings of the National Academy of Sciences of the United States of America **98:**10154-10159.
25. **Lamond, A. I., and D. L. Spector.** 2003. Nuclear speckles: a model for nuclear organelles. Nature Reviews Molecular Cell Biology **4:**605.
26. **Larue, R., K. Gupta, C. Wuensch, N. Shkriabai, J. J. Kessl, E. Danhart, L. Feng, O. Taltynov, F. Christ, G. D. Van Duyne, Z. Debyser, M. P. Foster, and M. Kvaratskhelia.** 2012. Interaction of the HIV-1 Intasome with Transportin 3 Protein (TNPO3 or TRN-SR2). Journal of Biological Chemistry **287:**34044-34058.
27. **Lee, E. g., A. Alidina, C. May, and M. L. Linial.** 2003. Importance of Basic Residues in Binding of Rous Sarcoma Virus Nucleocapsid to the RNA Packaging Signal. Journal of Virology **77:**2010-2020.
28. **Lee, K., Z. Ambrose, T. D. Martin, I. Oztop, A. Mulky, J. G. Julias, N. Vandegraaff, J. G. Baumann, R. Wang, W. Yuen, T. Takemura, K. Shelton, I. Taniuchi, Y. Li, J. Sodroski, D. R. Littman, J. M. Coffin, S. H. Hughes, D. Unutmaz, A. Engelman, and V. N. KewalRamani.** 2010. Flexible Use of Nuclear Import Pathways by HIV-1. Cell Host & Microbe **7:**221-233.
29. **Levin, A., Z. Hayouka, A. Friedler, and A. Loyter.** 2010. Transportin 3 and importin α are required for effective nuclear import of HIV-1 integrase in virus-infected cells. Nucleus **1:**422-431.
30. **Liu, H., and J. H. Naismith.** 2008. An efficient one-step site-directed deletion, insertion, single and multiple-site plasmid mutagenesis protocol. BMC Biotechnology **8:**91.

31. **Logue, E. C., K. T. Taylor, P. H. Goff, and N. R. Landau.** 2011. The Cargo-Binding Domain of Transportin 3 Is Required for Lentivirus Nuclear Import. J Virol **85:**12950-12961.
32. **Luban, J.** 2008. HIV-1 Infection: Going Nuclear with TNPO3/Transportin-SR2 and Integrase. Current Biology **18:**R710-R713.
33. **Matic, I., M. van Hagen, J. Schimmel, B. Macek, S. C. Ogg, M. H. Tatham, R. T. Hay, A. I. Lamond, M. Mann, and A. C. O. Vertegaal.** 2008. In vivo identification of human small ubiquitin-like modifier polymerization sites by high accuracy mass spectrometry and an in vitro to in vivo strategy. Mol Cell Proteomics **7:**132-144.
34. **Misteli, T., J. F. Cáceres, J. Q. Clement, A. R. Krainer, M. F. Wilkinson, and D. L. Spector.** 1998. Serine Phosphorylation of SR Proteins Is Required for Their Recruitment to Sites of Transcription In Vivo. The Journal of Cell Biology **143:**297.
35. **Nadaraia-Hoke, S., D. V. Bann, T. L. Lochmann, N. Gudleski-O'Regan, and L. J. Parent.** 2013. Alterations in the MA and NC domains modulate phosphoinositide-dependent plasma membrane localization of the Rous sarcoma virus Gag protein. J Virol **87:**3609-3615.
36. **Ngo, J. C. K., S. Chakrabarti, J.-H. Ding, A. Velazquez-Dones, B. Nolen, B. E. Aubol, J. A. Adams, X.-D. Fu, and G. Ghosh.** 2005. Interplay between SRPK and Clk/Sty Kinases in Phosphorylation of the Splicing Factor ASF/SF2 Is Regulated by a Docking Motif in ASF/SF2. Molecular Cell **20:**77-89.
37. **Ocwieja, K. E., T. L. Brady, K. Ronen, A. Huegel, S. L. Roth, T. Schaller, L. C. James, G. J. Towers, J. A. Young, S. K. Chanda, R. Konig, N. Malani, C. C. Berry, and F. D. Bushman.** 2011. HIV integration targeting: a pathway involving Transportin-3 and the nuclear pore protein RanBP2. PLoS Pathogens **7:**e1001313.
38. **Price, A. J., A. J. Fletcher, T. Schaller, T. Elliott, K. Lee, V. N. KewalRamani, J. W. Chin, G. J. Towers, and L. C. James.** 2012. CPSF6 Defines a Conserved Capsid Interface that Modulates HIV-1 Replication. PLOS Pathogens **8:**e1002896.
39. **Rice, B., R. Kaddis, M. Stake, T. Lochmann, and L. Parent.** 2015. Interplay between the alpharetroviral Gag protein and SR Proteins SF2 and SC35 in the nucleus. Frontiers in Microbiology **6**.
40. **Schaller, T., K. E. Ocwieja, J. Rasaiyaah, A. J. Price, T. L. Brady, S. L. Roth, S. Hué, A. J. Fletcher, K. Lee, V. N. KewalRamani, M. Noursadeghi, R. G. Jenner, L. C. James, F. D. Bushman, and G. J. Towers.** 2011. HIV-1 Capsid-Cyclophilin Interactions Determine Nuclear Import Pathway, Integration Targeting and Replication Efficiency. PLOS Pathogens **7:**e1002439.
41. **Scheifele, L. Z., R. Garbitt, J. D. Rhoads, and L. J. Parent.** 2002. Nuclear entry and CRM1-dependent nuclear export of the Rous sarcoma virus Gag polyprotein. Proceedings of the National Academy of Sciences **99:**3944-3949.
42. **Scheifele, L. Z., S. P. Kenney, T. M. Cairns, R. C. Craven, and L. J. Parent.** 2007. Overlapping Roles of the Rous Sarcoma Virus Gag p10 Domain in Nuclear Export and Virion Core Morphology. Journal of Virology **81:**10718.

43. **Scheifele, L. Z., E. P. Ryan, and L. J. Parent.** 2005. Detailed Mapping of the Nuclear Export Signal in the Rous Sarcoma Virus Gag Protein. Journal of Virology **79:**8732-8741.
44. **Shah, V. B., J. Shi, D. R. Hout, I. Oztop, L. Krishnan, J. Ahn, M. S. Shotwell, A. Engelman, and C. Aiken.** 2012. The Host Proteins Transportin SR2/TNPO3 and Cyclophilin A Exert Opposing Effects on HIV-1 Uncoating. Journal of Virology **87:**422-432.
45. **Shepard, P. J., and K. J. Hertel.** 2009. The SR protein family. Genome Biology **10:**242-242.249.
46. **Spector, D. L., and A. I. Lamond.** 2010. Nuclear Speckles. Cold Spring Harbor Perspectives in Biology **3:**a000646-a000646.
47. **Stake, M., D. Bann, R. Kaddis, and L. Parent.** 2013. Nuclear Trafficking of Retroviral RNAs and Gag Proteins during Late Steps of Replication. Viruses **5:**2767.
48. **Sutherland, H., and W. A. Bickmore.** 2009. Transcription factories: gene expression in unions? Nature Reviews Genetics **10:**457-466.
49. **Tsirkone, V. G., J. Blokken, F. De Wit, J. Breemans, S. De Houwer, Z. Debyser, F. Christ, and S. V. Strelkov.** 2017. N-terminal half of transportin SR2 interacts with HIV integrase. Journal of Biological Chemistry **292:**9699-9710.
50. **Valle-Casuso, J. C., F. Di Nunzio, Y. Yang, N. Reszka, M. Lienlaf, N. Arhel, P. Perez, A. L. Brass, and F. Diaz-Griffero.** 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.
51. **Verderame, M. F., T. D. Nelle, and J. W. Wills.** 1996. The membrane-binding domain of the Rous sarcoma virus Gag protein. Journal of Virology **70:**2664-2668.
52. **Zahler, A. M., W. S. Lane, J. A. Stolk, and M. B. Roth.** 1992. SR proteins: a conserved family of pre-mRNA splicing factors. Genes & Development **6:**837-847.
53. **Zhou, L., E. Sokolskaja, C. Jolly, W. James, S. A. Cowley, and A. Fassati.** 2011. Transportin 3 Promotes a Nuclear Maturation Step Required for Efficient HIV-1 Integration. PLOS Pathogens **7:**e1002194.

Figures


A.
MA
p2 p10
CA
NC
GFP
Gag.GFP
YFP
Gag.ΔNC.YFP
Gag.ΔMA5-86.GFP
Gag.ΔMA5-148.GFP
mCherry
RBD
NPC
CBD
mCherry.TNPO3
B.
Gag
Gag.ΔNC
Gag.ΔMA5-86
Gag.ΔMA5-148
TNPO3
C.
Gag
TNPO3
Gag.ΔNC
TNPO3
Gag.ΔMA5-86
TNPO3
Gag.ΔMA5-148
TNPO3
D.
% Nuclear Gag
0
10
20
30
40
50
*
*
WT Gag
WT Gag + TNPO3
Gag.ΔNC
Gag.ΔNC + TNPO3
Gag.ΔMA5-86
Gag.ΔMA5-86 + TNPO3
Gag.ΔMA5-148
Gag.ΔMA5-148 + TNPO3

626

Figure 1: Effects of TNPO3 expression on nuclear accumulation of Gag. A) Schematic representation of RSV Gag constructs used. Gag is composed of the domains: MA (matrix), p2, p10, CA (capsid), NC (nucleocapsid), and protease. Gag contains the entire coding region of Gag through the first seven amino acids of the PR domain. TNPO3 consists of a three domain structure including a N-terminal RanGTP binding domain (RBD), a nuclear pore complex (NPC) binding domain, and C-terminal cargo-binding domain (CBD). B) The localization of wildtype Gag is shown in the top row, followed by the localization of the Gag.ΔNC, Gag.$\Delta MA_{5\text{-}86}$ and Gag.$\Delta MA_{5\text{-}148}$. In the bottom row, shows the localization of mCherry.TNPO3 when overexpressed in cells. C) Cells expressing both the Gag proteins from (B) and mCherry.TNPO3 are shown. D) A graph plotting the percentage of nuclear Gag for each Gag protein shown with and without exogenous mCherry.TNPO3. At least 60 cells were analyzed from three independent experiments were analyzed for each condition, with the standard error of the mean represented by the error bars. " ***** " signifies a statistical significance ($p < 0.0001$) between the compared groups analyzed using unpaired Student's t-test. When mCherry.TNPO3 was added to the Gag expressing cells, only cells expressing both Gag and mCherry.TNPO3 were quantitated.

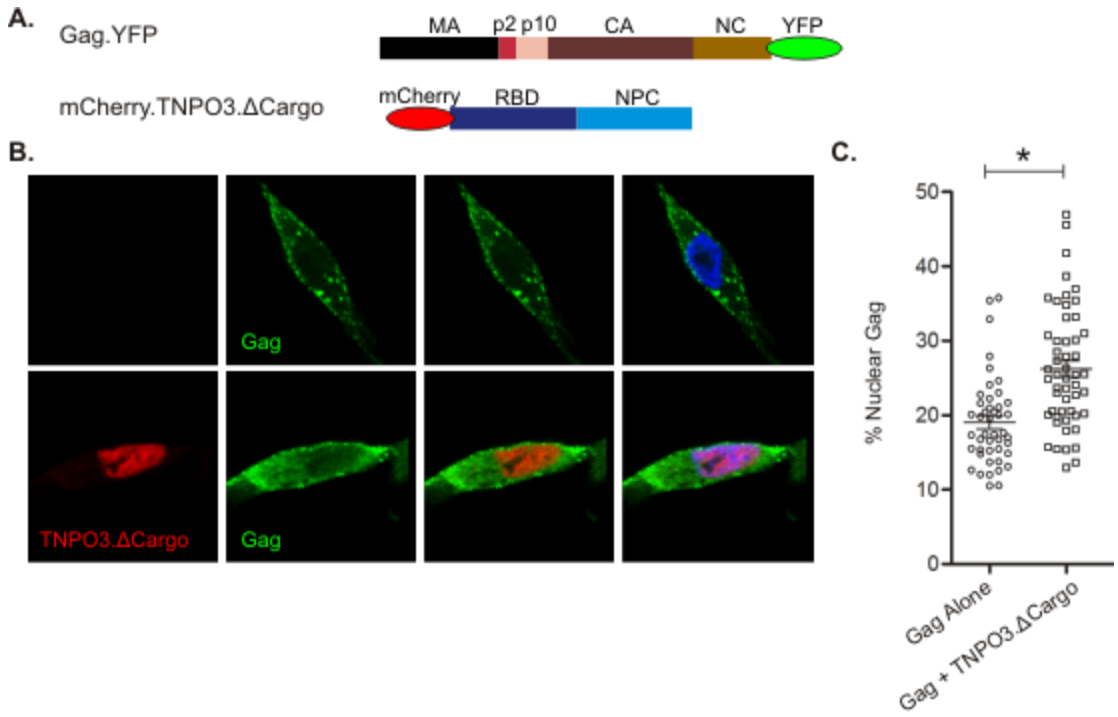
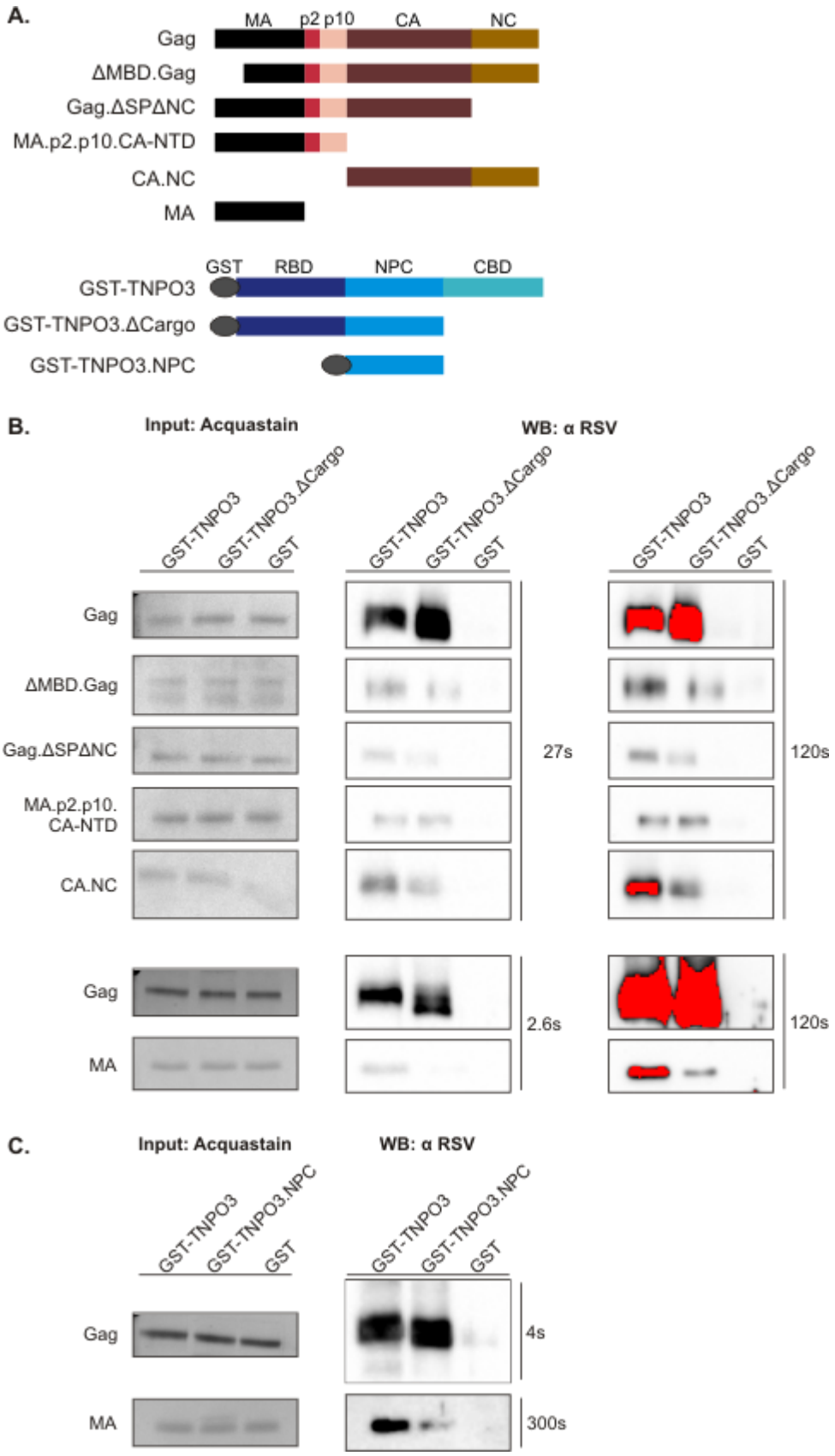
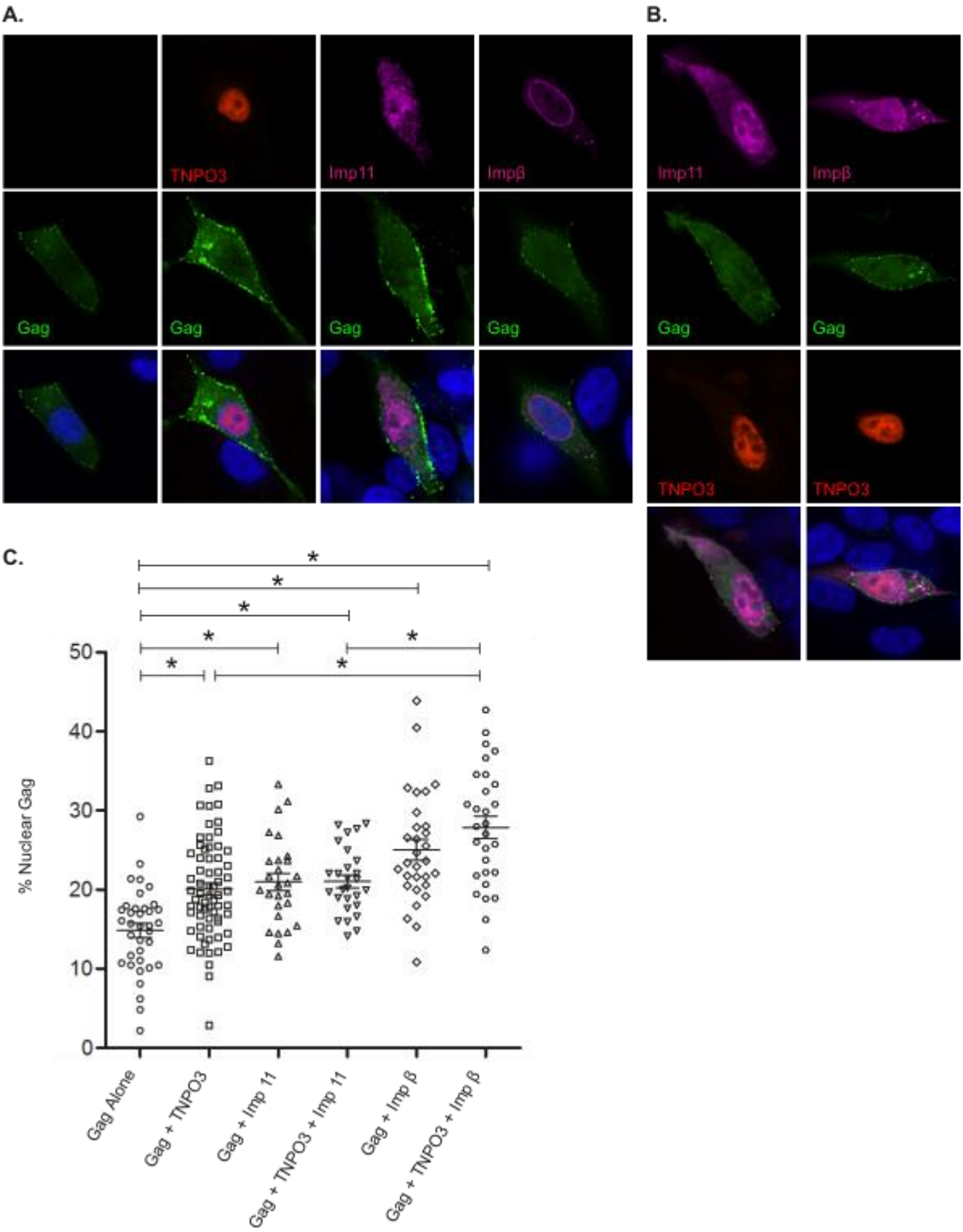


Figure 2: Gag nuclear accumulation with TNPO3 mutants. A) Schematic representation of the constructs used. TNPO3.ΔCargo has the entirety of the cargo domain and a small portion of the NPC domain deleted. B) The accumulation of wildtype Gag.YFP was examined when mCherry.TNPO3.ΔCargo wasexpressed in cells. C) A graph plotting the percentage of nuclear Gag with Gag expressed alone and with TNPO3.ΔCargo. At least 43 cells were analyzed for each condition from at least 3 independent experiments. The standard error of the mean is represented by the error bars with analysis done using the unpaired Student's t-test. " * " signifies $p < 0.0001$. Only cells expressing both Gag and mCherry.TNPO3.ΔCargo were quantitated.


A.
MA
p2
p10
CA
NC
Gag
ΔMBD.Gag
Gag.ΔSPΔNC
MA.p2.p10.CA-NTD
CA.NC
MA
GST
RBD
NPC
CBD
GST-TNPO3
GST-TNPO3.ΔCargo
GST-TNPO3.NPC
B.
Input: Acquastain
WB: α RSV
GST-TNPO3
GST-TNPO3.ΔCargo
GST
Gag
ΔMBD.Gag
Gag.ΔSPΔNC
MA.p2.p10.
CA-NTD
CA.NC
27s
120s
Gag
MA
2.6s
120s
C.
Input: Acquastain
WB: α RSV
GST-TNPO3
GST-TNPO3.NPC
GST
Gag
MA
4s
300s

654

Figure 3: *In vitro* affinity-tagged purifications of Gag and TNPO3 protein complexes. A) Schematic representation of constructs used. Gag contains the entire coding region of Gag through the first seven amino acids of the PR domain. SP stands for the spacer peptide located between CA and NC in Gag. NTD is the N-terminal domain of CA. TNPO3 consists of a three domain structure including a N-terminal RanGTP binding domain (RBD), a nuclear pore complex (NPC) binding domain, and C-terminal cargo-binding domain (CBD). The full-length TNPO3 protein is shown on the top, followed by a ΔCargo mutant that has the entirety of the cargo domain and a small portion of the NPC domain deleted. The TNPO3.NPC mutant contains most of the NPC domain and a portion of the C-terminal end of the RanGTP binding domain. B) In these affinity purifications, various Gag proteins were incubated with GST-TNPO3, GST-TNPO3.ΔCargo, or GST alone. On the left, a portion of the protein mixtures were removed before the incubation step and ran out on SDS-PAGE gel that was stained with Acquastain to visualize the proteins added to the affinity purification. On the right are gels of the elutions ran out on SDS-PAGE and analyzed by western blot using α-RSV antibodies exposed for different periods of time. The red indicates over saturation of signal. C) Same procedure as above except with GST-TNPO3.NPC incubated with Gag and the MA domain. A 300 second exposure was not obtained that contained both Gag and MA. At least 3 independent GST-tagged purifications were performed for each condition.


A.
TNPO3
Imp11
Impβ
Gag
Gag
Gag
Gag
B.
Imp11
Impβ
Gag
Gag
TNPO3
TNPO3
C.
% Nuclear Gag
0
10
20
30
40
50
Gag Alone
Gag + TNPO3
Gag + Imp 11
Gag + TNPO3 + Imp 11
Gag + Imp β
Gag + TNPO3 + Imp β

675

676

Figure 4: Effects of multiple import factors on Gag nuclear accumulation. A) Visualization of cellular localization of wildtype Gag.YFP (green) alone (first column) and with exogenous individual import factors, mCherry.TNPO3 (red: second column), HA-importin-11 (magenta: third column), and HA-importin-β (magenta: fourth column). B) Visualization of cellular localization of wildtype Gag with exogenous importin-11 and TNPO3 (first column), and importin-β and TNPO3 (second column). C) A graph displaying the percentage of Gag either with no exogenous import factors, with TNPO3, importin-11 and importin-β individually, and then TNPO3 with either importin-11 or importin-β. At least 27 cells were analyzed from two independent experiments for each condition with the standard error of the mean represented by the error bars. " ***** " signifies a statistical significance ($p < 0.0001$) between the compared groups. Group comparisons were analyzed using unpaired Student's t-tests. For conditions of Gag with an import factor, only cells expressing Gag and the import factor(s) were quantitated.