Viral protein X unlocks the nuclear pore complex through a human Nup153-dependent pathway to promote nuclear translocation of the lentiviral genome

Satya Prakash Singh[a], Sebastian Raja[a], and Sundarasamy Mahalingam[a,b,*]
[a]Laboratory of Molecular Cell Biology and [b]National Cancer Tissue Biobank, Bhupat and Jyoti Mehta School of Biosciences, Department of Biotechnology, Indian Institute of Technology-Madras, Chennai 600 036, India

ABSTRACT **Simian immunodeficiency virus (SIV) and human immunodeficiency virus 2 (HIV-2) display unique ability to infect nondividing target cells. Viral protein X (Vpx) of HIV-2/SIV is known to be involved in the nuclear import of viral genome in nondividing cells, but the mechanism remains poorly understood. In the present investigation for the first time we provide evidence that Vpx of $SIV_{smPBj1.9}$ physically interacts with human nucleoporin 153 (Nup153), which is known to provide a docking site for protein-cargo complexes at the nuclear pore complex (NPC). Results from superresolution-structured illumination microscopy studies reveal that Vpx interaction with NPC-associated Nup153 is critical for its efficient nuclear translocation. Virion-associated MAPK/ERK-2–mediated phosphorylation of Vpx plays a critical role in its interaction with human Nup153 and this interaction was found to be evolutionarily conserved in various SIV isolates and HIV-2. Interestingly, MAPK/ERK-2 packaging defective SIV failed to promote the efficient nuclear import of viral genome and suggests that MAPK/ERK-2–mediated Vpx phosphorylation is important for its interaction with Nup153, which is critical for lentiviruses to establish infection in nondividing target cells. Together, our data elucidate the mechanism by which Vpx orchestrates the challenging task of nuclear translocation of HIV-2/SIV genome in nondividing target cells.**

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INTRODUCTION

The early stage of lentiviral replication involves reverse transcription of the viral RNA genome in the cytoplasm of the host cell. The newly synthesized linear double-stranded viral cDNA together with viral and host cell proteins forms preintegration complex (PIC). Nuclear translocation of PIC is critical for the integration of viral genome into the host chromosome and is one of the key steps during early events of the virus life cycle (Bowerman *et al.*, 1989; Brown *et al.*, 1989; Farnet and Haseltine, 1990). However, nuclear translocation of the PIC in nondividing primary target cells is challenged by an intact nuclear envelope that serves as a physical boundary between nucleus and cytoplasm. Moreover, the bigger size of the viral PIC (stock diameter ≃56 nm) restricts its unconstrained dispersion through the central channel of the nuclear pore complex (NPC) (Miller *et al.*, 1997). Infection of Gammaretroviruses such as Moloney murine leukemia virus and feline leukemia virus requires dissociation of the nuclear envelope during mitosis for the direct access of viral DNA to the host chromosome (Roe *et al.*, 1993; Katz *et al.*, 2005). On the other hand, lentiviruses such as human immunodeficiency viruses (HIV) and simian immunodeficiency viruses (SIV) have the unique ability to infect nondividing host cells (Weinberg *et al.*, 1991; Lewis and Emerman, 1994; Zhang *et al.*, 1999). The PIC of HIV-1 consists of

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*Address correspondence to: Sundarasamy Mahalingam (mahalingam@iitm.ac.in).

Abbreviations used: CA, capsid; DAPI, 4′,6-diamidino-2-phenylindole; FBS, fetal bovine serum; HIV, human immunodeficiency virus; IN, integrase; MA, matrix; NC, nucleocapsid; NPC, nuclear pore complex; Nup153, nucleoporin 153; PIC, preintegration complex; PMSF, phenylmethylsulfonyl fluoride; RT, reverse transcriptase; SIV, simian immunodeficiency virus; SR-SIM, superresolution-structured illumination microscopy; VLP, virus-like particle; Vpr, viral protein R; Vpx, viral protein X; WT, wild type.

proviral DNA and viral proteins, namely, reverse transcriptase (RT), integrase (IN), matrix (MA), nucleocapsid (NC), and viral protein R (Vpr) (Farnet and Haseltine, 1991; Suzuki and Craigie, 2007). Extensive studies on each of these PIC-associated viral elements have suggested their role in the nuclear import of the HIV-1 genome (Bukrinsky *et al.*, 1993; Heinzinger *et al.*, 1994; Haffar *et al.*, 2000; Dvorin *et al.*, 2002; Ikeda *et al.*, 2004; Yamashita *et al.*, 2007). Although most of the capsid (CA) molecules are separated during the viral core uncoating process in the cytoplasm, a significant amount of CA was shown to be associated with viral PICs at NPC (Matreyek and Engelman, 2011; Schaller *et al.*, 2011; Francis and Melikyan, 2018). Collectively, these studies suggest that multiple viral determinants contribute to the nuclear import of HIV-1 PIC in nondividing target cells. However, compared with HIV-1, nuclear import of HIV-2/SIV PIC is not reported to be following a similar mechanism. HIV-2/SIV PIC differs from that of HIV-1 by having an additional factor, viral protein X (Vpx) (Henderson *et al.*, 1988; Kappes *et al.*, 1988). Furthermore, Vpx but not Vpr of HIV-2/SIV has been shown to be critical for the proficient nuclear import of the viral genome (Ueno *et al.*, 2003). In vivo studies in macaques indicate that Vpr-deleted viruses behaved similar to wild type while viruses lacking Vpx were extremely flawed in promoting viral pathogenesis (Gibbs *et al.*, 1995; Hirsch *et al.*, 1998). Vpx contains multiple noncanonical NLS sequences and studies on infection of monocyte and monocyte-derived macrophages with SIV_{sm} and HIV-2 indicated that Vpx was sufficient for efficient nuclear translocation of HIV-2/SIV PIC (Mahalingam *et al.*, 2001; Belshan *et al.*, 2006; Singhal *et al.*, 2006a; Goujon *et al.*, 2008).

Considering the nuclear import of the HIV-2/SIV genome is one of the critical events during early steps in the virus life cycle, it is important to explore the mechanism(s) of its nuclear entry during virus infection. Active nuclear transport of macromolecules across NPC is facilitated by a selective barrier composed of a number of nucleoporins that provide the docking sites for cargo-protein complexes at NPC (Alber *et al.*, 2007). A genome-wide RNA interference screening suggested the requirement of Nup98, Nup358, and nucleoporin 153 (Nup153) for the nuclear translocation of the HIV-1 genome in nondividing cells (Ebina *et al.*, 2004; Brass *et al.*, 2008; König *et al.*, 2008; Zhou *et al.*, 2008). In particular, knockdown of Nup153 resulted in poor viral replication in target cells (Matreyek and Engelman, 2011). Moreover, HIV-1 PIC-associated IN, CA, and Vpr proteins were reported to have coordinated interactions with Nup153 (Varadarajan *et al.*, 2005; Woodward *et al.*, 2009; Matreyek *et al.*, 2013). Nup153 is a NPC-associated nuclear basket protein, known to be involved in importin alpha/beta-mediated nuclear import of several cellular proteins (Ullman *et al.*, 1999; Ogawa *et al.*, 2012; Duheron *et al.*, 2014). The present investigation provided evidence that HIV-2/SIV Vpx physically interacts with human Nup153 and the region between amino acid residues 61 and 80 in the C-terminal domain of Vpx is critical for its interaction with Nup153. Interestingly, MAPK/ERK-2–mediated Vpx phosphorylation is critical for its interaction with Nup153. Viruses defective with MAPK/ERK-2 packaging were found to be impaired in nuclear import of the viral genome in G2/M-arrested cells. Furthermore, data from superresolution-structured illumination microscopy (SR-SIM) studies revealed that Vpx interaction with Nup153 resulted in the alteration at NPC during nuclear translocation. Together, our results suggest that the interaction between lentiviral Vpx and human Nup153 is critical for translocating the viral genome into the nucleus for the establishment of viral infection in nondividing target cells.

RESULTS

$SIV_{smPBj1.9}$ Vpx physically interacts with human Nup153

HIV-1 Vpr, a homologous protein of HIV-2/SIV Vpx, is known to be involved in the nuclear translocation of HIV-1 PIC and found to have direct interaction with human Nup153 (Fouchier *et al.*, 1998; Varadarajan *et al.*, 2005). Tertiary structure alignment of Vpr and Vpx retrieved from the RCSB protein database shows both proteins have highly conserved structural domains (Supplemental Figure S1, A and B). Reports from others and our laboratory have demonstrated that Vpx is localized in the nuclear compartment and is indispensable for the nuclear translocation of the HIV-2/SIV genome in primary nondividing target cells (Hirsch *et al.*, 1998; Mahalingam *et al.*, 2001; Belshan *et al.*, 2006). However, the mechanism by which Vpx promote the nuclear translocation of the viral genome remains unknown. Toward understanding this, we first tested whether Vpx can interact with endogenous human Nup153. Vpx and Vpr of $SIV_{smPBj1.9}$ (PBj1.9 isolate from Sooty Mangabey, referred as SIVsm) as fusion with GFP were ectopically expressed in HEK293T cells and analyzed for their physical interaction with endogenous Nup153. Results in Figure 1A indicate that Vpx (lane 3) but not Vpr (lane 2) of SIV_{sm} specifically interacts with Nup153. Further, to confirm this interaction, reciprocal coimmunoprecipitation was performed with cell lysates containing Nup153-GFP and Flag-Vpx using anti-Flag antibodies followed by Western blot with anti-GFP antibodies. Results in Supplemental Figure S1C indicate that Nup153-GFP but not GFP was able to coimmunoprecipitated with Vpx (lane 1). Recent reports suggest that Vpx interacts with the components of classical nuclear import pathways (Singhal *et al.*, 2006a,b). Considering the fact that Nup153 provides docking sites for protein-cargo complexes of the classical nuclear import pathway, we next investigated whether Vpx interaction with Nup153 is independent of its association with the classical nuclear import pathway. Toward this, transport receptors importin-alpha and importin-beta were depleted using gene-specific small interfering RNAs (siRNAs) in GFP–Vpx-transfected HEK293T cells and the cell lysates were subjected to coimmunoprecipitation using anti-Nup153 antibodies followed by Western blot with the anti-GFP antibodies. Interestingly, results in Figure 1B indicate that Vpx efficiently interacts with Nup153 under importin–alpha- and importin–beta-depleted conditions (lane 2). Knockdown efficiencies of importin-alpha and importin-beta were determined by Western blot using specific antibodies. Taken together, these results suggest that human Nup153 is a bona fide interacting partner for SIV_{sm} Vpx.

It is known that Vpx interacts with SAMHD1 and this interaction is critical for Vpx-mediated SAMHD1 degradation during virus infection. We next tested whether SAMHD1 alters the interaction between Vpx and human Nup153. Toward this, cell lysates containing Flag-SAMHD1 and GFP-Vpx were coimmunoprecipitated with anti-GFP antibodies followed by Western blot with anti-Nup153 and anti-Flag antibodies. Results indicate that both SAMHD1 and Nup153 coprecipitated with Vpx (Supplemental Figure S1D). These results suggest that SAMHD1 is not competing with Nup153 for Vpx interaction and Vpx encode distinct binding domains for Nup153 and SAMHD1 as supported by the existence of trimeric Vpx-Nup153-SAMHD1 complex. Furthermore, these results indicate that Nup153- and SAMHD1-binding domains in Vpx are nonoverlapping.

To identify the domain(s) in Vpx is critical for its interaction with Nup153, various GFP-fused Vpx deletion mutants were generated (Figure 1C). All Vpx variants were sequence verified and transiently expressed in HEK293T cells. Cell lysates containing indicated Vpx variants were subjected to coimmunoprecipitation using anti-Nup153 antibodies. Western blot analysis of immunoprecipitated

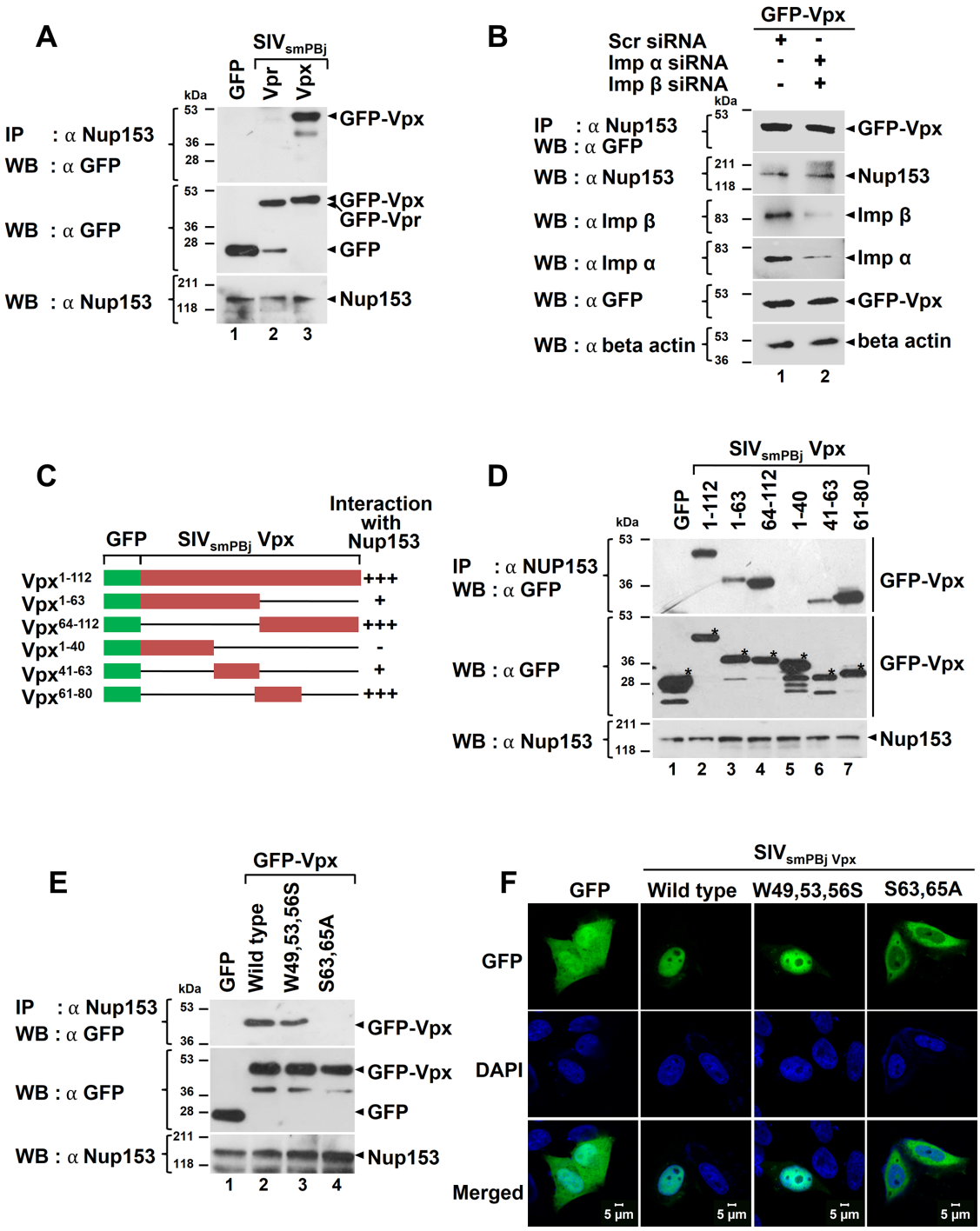

FIGURE 1: Interaction of SIV_{sm} Vpx with human Nup153. (A) HEK293T cell lysates containing Vpr-GFP and GFP-Vpx proteins of SIV_{sm} were coimmunoprecipitated with anti-Nup153 antibody followed by Western blot with the anti-GFP antibody. Results indicate that Vpx specifically interacts with human Nup153. (B) Importin-alpha and importin-beta were depleted using gene-specific siRNAs in GFP–Vpx-expressing HEK293T cells. Cell lysates were subjected to coimmunoprecipitation using anti-Nup153 antibody followed by Western blot with the anti-GFP antibody. (C) Schematic diagram of Vpx mutants and their interaction status with Nup153. (D) HEK293T cell lysates containing indicated variants of Vpx were subjected to coimmunoprecipitation with anti-Nup153 antibody followed by Western blot using anti-GFP antibody. Results clearly indicate that the region between amino acids 61 and 80 of Vpx is required for its interaction with Nup153. (E) Immunoprecipitation was performed with HEK293T cell lysates containing Vpx variants using anti-Nup153 antibodies followed by Western blot with anti-GFP antibodies. Indicated Vpx variants were generated by exchanging the conserved tryptophan residues at positions 49, 53, and 56 with serines ($Vpx^{W49,53,56S}$) and serine residues at positions 63 and 65 with alanines ($Vpx^{S63,65A}$) by site-directed mutagenesis using SIVsm Vpx as template. (F) Subcellular distribution of GFP-tagged wild type and mutant Vpx proteins. HeLa cells expressing indicated that GFP-Vpx variants were fixed, stained with DAPI, and imaged with LSM710 laser-scanning confocal microscope (Carl Zeiss, Germany). All the experiments were repeated three times with similar results.

complexes with anti-GFP antibodies clearly indicates that the region between residues 61 and 80 of Vpx is critical for its interaction with Nup153 (Figure 1D, top panel, lane 7). Furthermore, to identify specific residues that are involved in Vpx interaction with Nup153, conserved residues within 61–80 regions of Vpx were exchanged with alanine, and the resultant Vpx variants were expressed in HEK293T cells. Cell lysates containing GFP-Vpx variants were subjected to coimmunoprecipitation with anti-Nup153 antibodies followed by Western blot analysis with the anti-GFP antibodies. Results in Figure 1E suggest that conserved serine residues at positions 63 and 65 within 61–80 regions in Vpx are critical for its interaction with Nup153 (top panel, lane 4). Since the $Vpx^{S63,65A}$ mutant failed to interact with Nup153, we next examined its ability to translocate into the nucleus. Results from the immunofluorescence analysis of GFP–Vpx-expressing HeLa cells indicate that the $Vpx^{S63,65A}$ mutant protein was localized in the cytoplasm; in contrast, the $Vpx^{W49,53,55S}$ mutant interacts with Nup153 like wild-type Vpx (Figure 1E, lanes 2 and 3) retained WT nuclear localization (Figure 1F). These results suggest that serine residues at 63 and 65 in SIV_{sm} Vpx are critical for its interaction with Nup153 and subsequent nuclear translocation. To identify the domain within Nup153 that is essential for Nup153's interaction with Vpx, variants of Nup153 were generated as HA-tag fusion. Nup153 variants were coexpressed with GFP-Vpx in HEK293T cells and the cell lysates were subjected to coimmunoprecipitation with anti-GFP antibodies followed by Western blot using anti-HA antibodies. Results in Supplemental Figure S1E indicate that the region between amino acids 610 and 869 of Nup153 is essential for its interaction with Vpx (top panel, lane 2) and suggest the specific interaction of human Nup153 with SIV_{sm} Vpx.

Nup153-dependent nuclear translocation of SIV_{sm} Vpx

Results from immunoprecipitation, mutagenesis, and subcellular localization analyses clearly established that SIVsm Vpx physically interacts with human Nup153. We next tested whether nuclear translocation of Vpx is dependent on Nup153. Toward this end, the cellular distribution of Vpx and Nup153 at the nuclear envelope of HeLa cells was analyzed. GFP-Vpx was found to be transported into the nucleus; however, few Vpx oligomeric molecules formed puncta and colocalized with Nup153 at the nuclear envelope. Since the size of individual NPCs (approximately 120 nm in diameter) (Farnet and Haseltine, 1991; Maimon *et al.*, 2012) falls well below the resolution limit of conventional light microscopy (resolution limit of 200–300 nm), SR-SIM technique (Schermelleh *et al.*, 2010) was used to examine the colocalization pattern of Vpx and Nup153 (Supplemental Figure S2, A and B). HeLa cells expressing GFP and GFP-Vpx were fixed with paraformaldehyde and stained for endogenous Nup153 (red) and lamin (cyan to differentiate nuclear and cytoplasmic compartments) followed by nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI). GFP was found to be distributed homogeneously both in the nuclear and in the cytoplasmic compartments (Figure 2A). In support with the previous reports (Pancio *et al.*, 2000; Rajendra Kumar *et al.*, 2003; Goujon *et al.*, 2008; Singh *et al.*, 2019) on the localization of Vpx, we observed differential subcellular localization pattern of Vpx despite predominant nuclear localization. In particular, GFP-Vpx was found to be concentrated at the nuclear envelope of HeLa cells (Figure 2B), which suggests a potential interaction of Vpx with the components of NPC. Furthermore, we have analyzed 500 individual regions of the nuclear envelope from 100 Vpx-expressing cells and noticed that GFP-Vpx colocalized (83%) with Nup153 irrespective of the size of Vpx puncta (Supplemental Figure S2, C and D). Fluorescence intensity profile at the nuclear membrane along the X-Y plan indicates that GFP does not colocalize with Nup153 (Supplemental Figure S2E). However, the peak intensity of lamin across the nuclear envelope of GFP–Vpx-expressing HeLa cells suggests that both Vpx and Nup153 are colocalized at the nuclear membrane (Supplemental Figure S2E). SR-SIM analysis of $Vpx^{S63,65A}$, which is defective for Nup153 interaction, showed predominant cytoplasmic localization (Supplemental Figure S3A). Furthermore, the colocalized regions of Vpx puncta and Nup153 at the nuclear envelope suggest that both Vpx and Nup153 are in close proximity at NPC (Figure 2B). To further confirm the requirement of Nup153 for the efficient nuclear translocation of Vpx, endogenous Nup153 was depleted using specific short hairpin RNA (shRNA) in RFP–Vpx-expressing HeLa cells. As expected, compared with the scrambled shRNA-transfected cell, nuclear localization of Vpx was severely altered in Nup153-depleted HeLa cells (Figure 2, C and D). The efficiency of shRNA-mediated Nup153 knockdown was determined by Western blot analysis using anti-Nup153 antibodies (Supplemental Figure S3B, lane 2). Collectively, these results provide evidence that Nup153 is indeed an essential factor for efficient nuclear translocation of Vpx.

Vpx colocalization with Nup153 at the nuclear envelope facilitates its nuclear translocation

Results from the Nup153 knockdown experiments clearly indicated the Nup153 dependency on Vpx nuclear translocation. We next used 3D SR-SIM to understand the mechanism of Vpx and Nup153 colocalization at NPC during nuclear translocation. HeLa cells expressing GFP-Vpx were immune-stained with anti-Nup153 antibodies, and the multicolor 3D-SIM images were acquired as described in *Materials and Methods*. Optical sectioning of Vpx colocalization with Nup153 at NPC (Figure 3A, Z-stacks 1–16) indicates the respective positions of Vpx and Nup153 across the nuclear envelope. Three-dimensional volumetric projection analysis using Imaris software reveals that Vpx seems to be submerged within Nup153 without causing any apparent alteration in the regular arrangement of Nup153 at the nuclear envelope (Figure 3A, 3D model projection). Intensity profile of the corresponding Z-plane (white dotted line) in the contact region of Vpx (green) with Nup153 (red) indicates the relative positions of Vpx and Nup153 in each Z-stack. Initial peak intensities of Vpx indicate its variable distribution near the nuclear envelope (Figure 3A, Z-stacks 1–3). However, on close proximity with Nup153, Vpx (yellow region) peak intensity perfectly superimposed with Nup153, suggesting a coordinated arrangement of Vpx at NPC (Figure 3A, Z-stacks 4–8). Diminished peak intensity of Vpx in the follow-up Z-planes (Figure 3A, Z-stacks 9–16) indicates that Vpx dispersal into the nucleoplasm. Furthermore, 2.5D intensity projection analysis delineates the nuclear envelope boundary as a regular pattern of Nup153 at NPC with perfect colocalization of Vpx (Supplemental Figure S4A). To clearly visualize the nuclear entry of Vpx, a live cell imaging experiment was performed with HeLa cells expressing GFP-Vpx as described in *Materials and Methods*. Results in Figure 3B and Supplemental Figure S4B (corresponding Supplemental Movie S7, A and B) suggest the conspicuous nuclear entry of Vpx punctum at different time intervals. Together, these results suggest that Vpx colocalization with Nup153 at NPC is critical for its efficient entry into the nucleus.

Interaction between Vpx and Nup153 is evolutionarily conserved among various HIV-2/SIV isolates

To find out whether the interaction of Vpx with human Nup153 is evolutionarily conserved, the amino acid residues within the 61–80 region of SIV_{sm} Vpx were compared with Vpx from different HIV-2

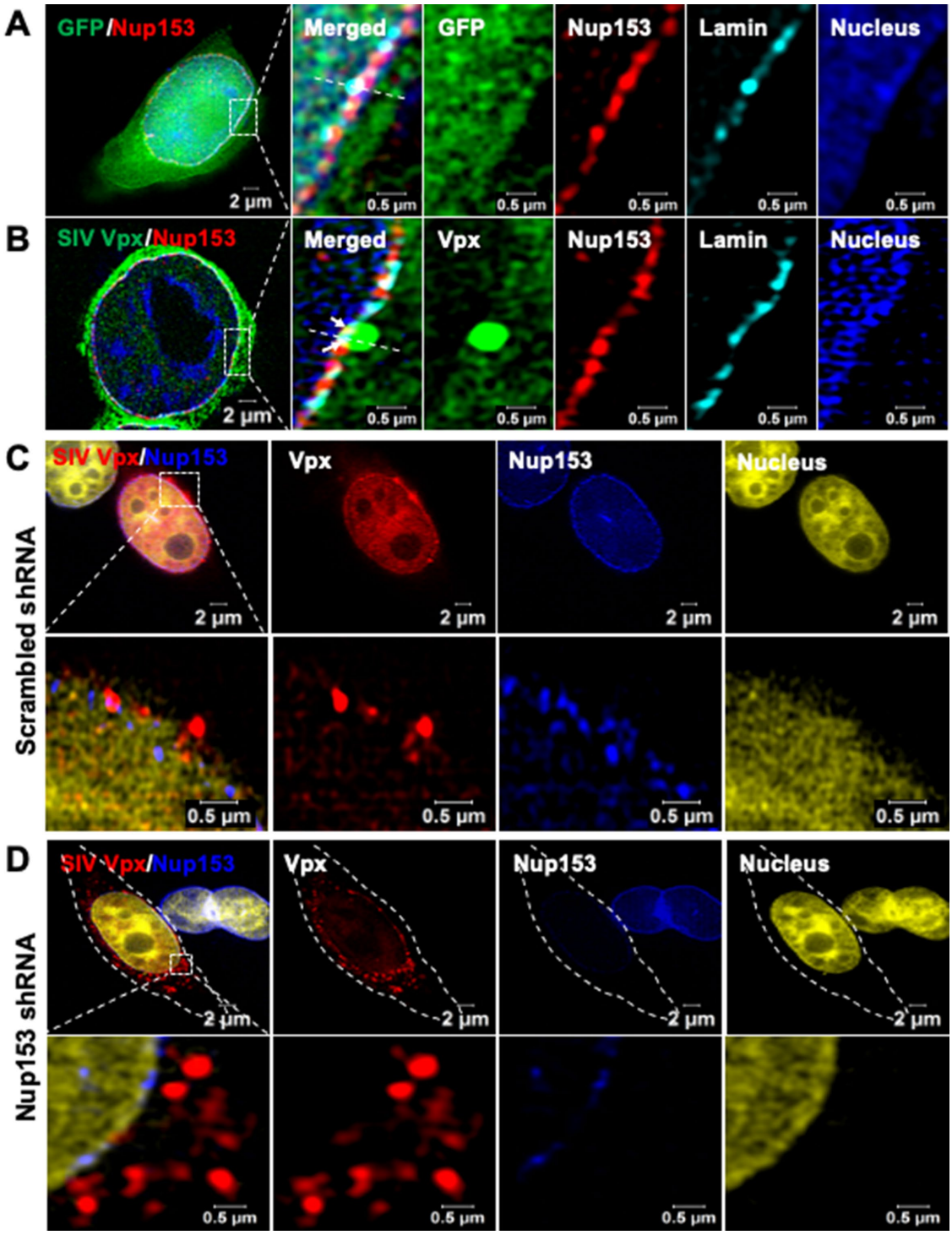

FIGURE 2: Human Nup153-dependent nuclear transport of SIVsm Vpx. HeLa cells expressed with GFP (A) and (B) GFP-Vpx were imaged with SR-SIM as described in *Materials and Methods*. Inset is the magnified nuclear membrane showing the colocalized region of SIVsmVpx with Nup153. Green, GFP-Vpx; red, Nup153; cyan, Lamin; blue, nucleus. Scrambled (C) or Nup153-specific shRNA (D) was cotransfected with RFP-Vpx in HeLa cells. Depletion of endogenous Nup153 abrogated the nuclear translocation of Vpx. Red, RFP-Vpx; blue, Nup153; and yellow, nucleus. All experiments were repeated three times with similar results.

and SIV isolates. As expected, serine residues within the 61–80 region (with reference to SIV_{sm}) from various Vpx were found to be highly conserved (Figure 4A). Results from sequence alignment analysis together with subcellular localization patterns of SIV_{sm} Vpx variants (Figure 1F) suggest that serine residues within the 61–80 region might play a critical role in Nup153 interaction dependent nuclear localization of Vpx. To understand further, Vpx from relatively distinct lineage of HIV-2/SIV isolates were selected; $HIV\text{-}2_{ISY}$ (having no variation within the 61–80 region with reference to SIV_{sm}), SIV_{rcm} (SIV isolate from Red-capped Mangabey; having E61, which

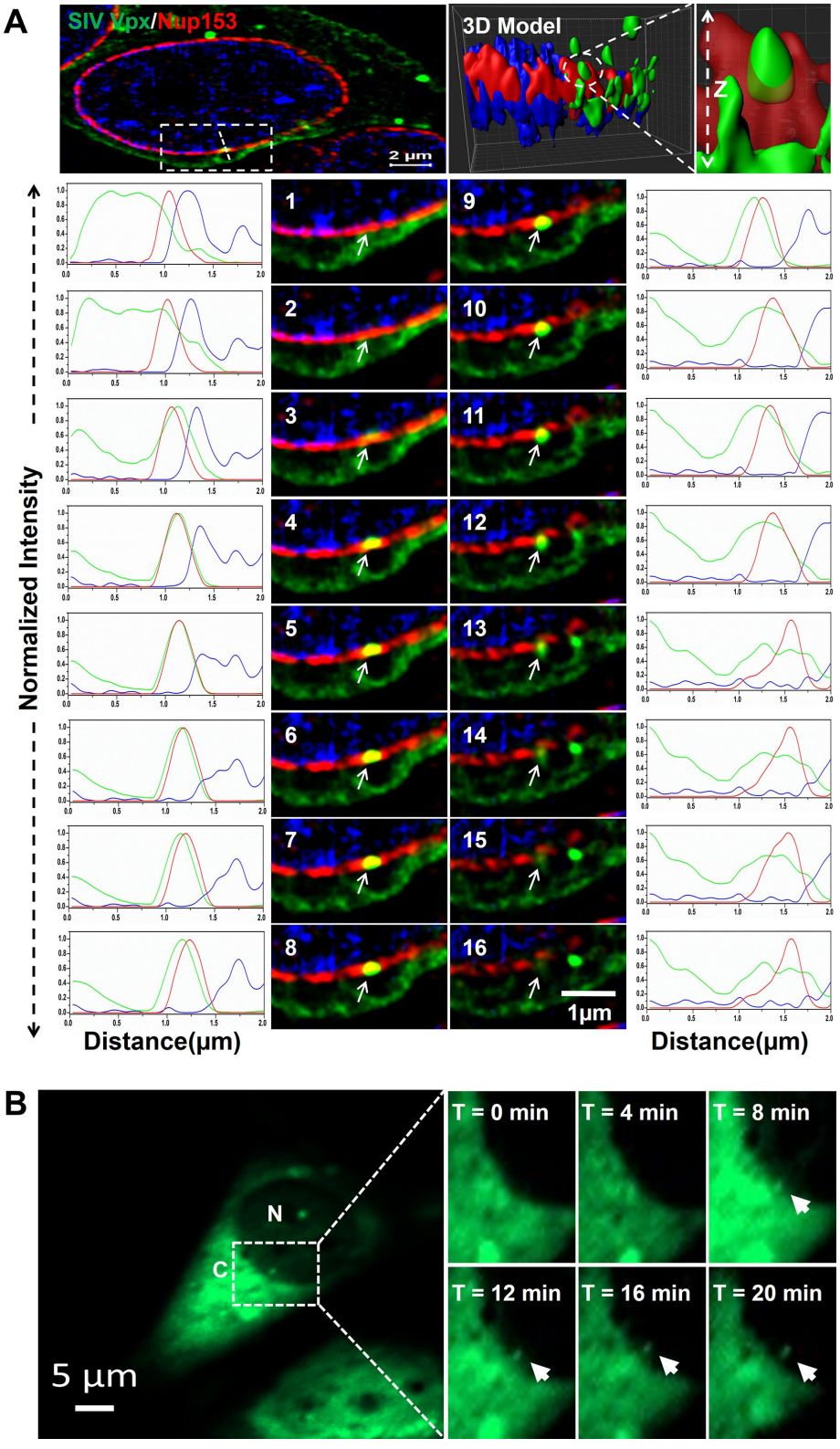

FIGURE 3: SIVsm Vpx colocalization with Nup153 is critical for its translocation into the nucleus. (A) SIM images of HeLa cells indicate the localization of GFP-Vpx (inset) at the nuclear membrane (indicated by a white dashed line). Z-stack analysis of nuclear membrane indicates the region of GFP-Vpx (yellow) is colocalized with Nup153 (red). Successive Z-stacks images captured across the *z*-axis indicate the position of Vpx and Nup153 across NPC. The graph plotted using normalized fluorescence intensity against distance (µm) is represented in all Z-stacks. Total thickness of the cell was about ~5 µm and each Z-stack with 110 nm in thickness was captured with an exposure time of 100 ms. The 3D volumetric reconstruction of Vpx localization was generated using Imaris software. (B) Dynamics of Vpx nuclear translocation was imaged by fluorescence microscope (Carl Zeiss, Germany) at different time intervals in HeLa cells. All experiments were repeated three times with similar results. Representative images indicate that Vpx puncta localized at the nuclear periphery and gradually translocated into the nucleus.

is S65 in SIV_{sm}), and SIV_{mnd2} (SIV isolate M14 from Mandrill; having T59 and N61, which are S63 and 65 in SIV_{sm}) (Peeters and Courgnaud, 2002). Expression constructs of Vpx from SIV_{rcm}, SIV_{mnd2}, and HIV-2_{ISY} were generated as GFP fusion and expressed in HEK293T cells. Cell lysates containing indicated Vpx variants were subjected to coimmunoprecipitation with anti-Nup153 antibodies followed by Western blot analysis using anti-GFP antibodies. HIV-2_{ISY} Vpx (both serines are conserved) showed an interaction with Nup153 as like Vpx of SIV_{sm} (Figure 4B, top panel, lanes 2 and 3), whereas Vpx of SIV_{rcm}, containing serine residue at position 59 showed poor interaction with Nup153 (Figure 4B, top panel, lane 4). Interestingly, no interaction was detected between Vpx of SIV_{mnd2} (contained no conserved serine residues) and Nup153 (Figure 4B, top panel, lane 5) despite equal expression levels that were observed (Figure 4B, middle panel, lanes 2–5). To further confirm the importance of serine residues on Vpx interaction with Nup153, Vpx variants of SIV_{rcm} and SIV_{mnd2} were generated (Supplemental Figure S4C). WT and indicated Vpx variants of SIV_{sm}, SIV_{rcm}, and SIV_{mnd2} were expressed in HEK293T cells and were subjected to coimmunoprecipitation as described above. As expected, similar to $Vpx^{S63,65A}$ of SIV_{sm} (Figure 4C, top panel, lane 2), replacement of serine 59 with alanine impaired the interaction of mutant SIV_{rcm} Vpx with Nup153 (Figure 4C, top panel, lane 4). Interestingly, Vpx variant of SIV_{mnd2} with serines at positions 59 and 61 showed interaction with Nup153 despite no interaction noticed for WT Vpx with Nup153 (Figure 4C, top panel, lanes 5 and 6), suggesting a "gain of function" for SIV_{mnd2} Vpx variant. Together, these results suggest that the conserved serine residues are critical for lentiviral Vpx interaction with Nup153. We next addressed whether the introduction of serine residues altered the subcellular localization Vpx variants. WT and indicated variants of Vpx were transiently expressed in HeLa cells and the results from the cellular localization analysis indicate that an exclusive nuclear localization of WT Vpx of SIVrcm, in contrast to the diffused nuclear (intense) and cytoplasmic localization pattern of SIVrcmVpx^{S59A} (Figure 4D), failed to

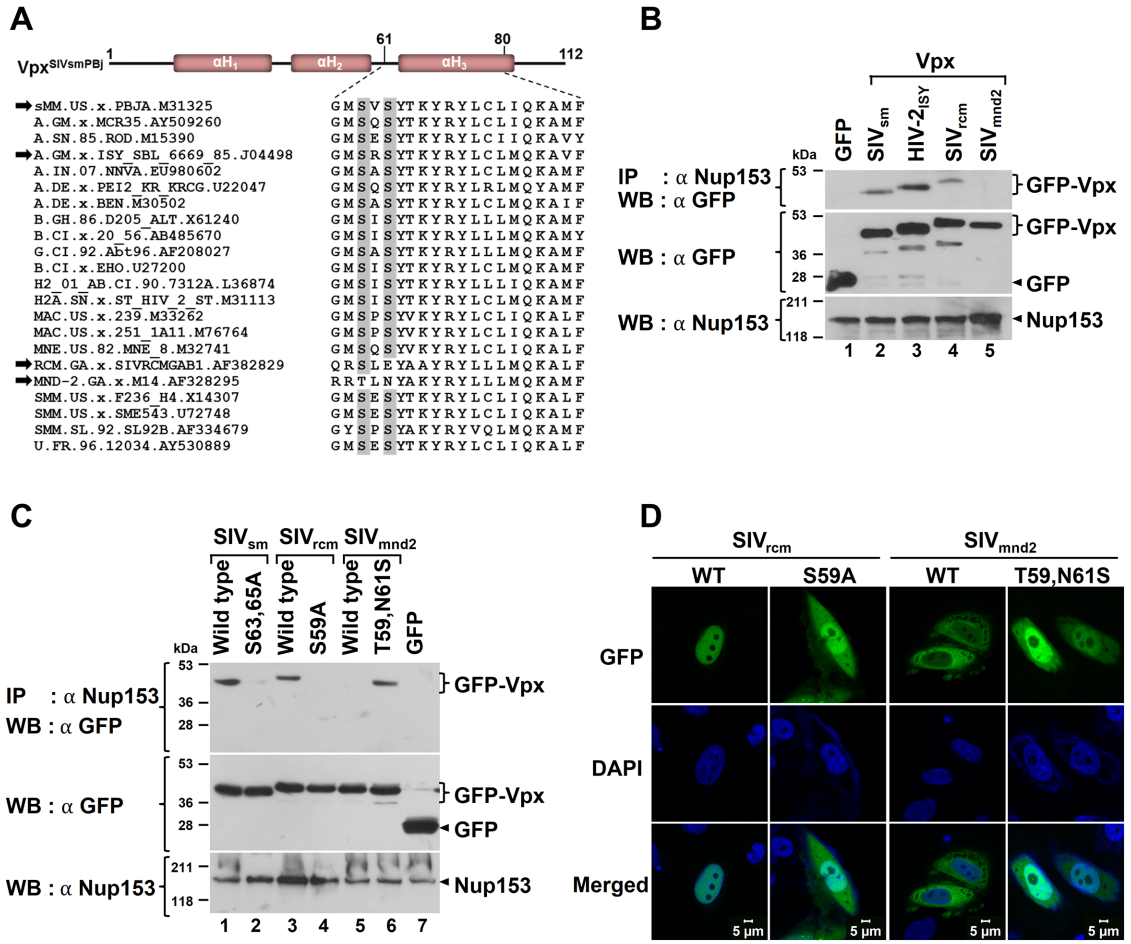

FIGURE 4: Interaction between lentiviral Vpx and human Nup153 is evolutionarily conserved. (A) Sequence alignment of amino acid residues within the 61–80 region of Vpx from various HIV-2/SIV strains obtained from the Los Alamos HIV sequence database indicates that serine residues are conserved in lentiviral Vpx proteins. Arrows indicate the Vpx proteins were selected from HIV-2 and various SIV isolates for Nup153 interaction. (B) GFP-tagged Vpx of SIV_{sm}, $HIV\text{-}2_{ISY}$, SIV_{rcm}, and SIV_{mnd2} were expressed in HEK293T cells and the cell lysates were subjected to coimmunoprecipitation with anti-Nup153 antibody followed by Western blot with anti-GFP antibody. Results indicate a weak interaction of SIV_{rcm} Vpx whereas Vpx of SIV_{mnd2} failed to interact with Nup153. (C) Vpx variants containing HEK293T cell lysates were subjected to coimmunoprecipitation with anti-Nup153 antibody followed by Western blot using anti-GFP antibody. Results indicate that Vpx variants from SIV_{sm} and SIV_{rcm} failed to interact with Nup153; in contrast, Vpx variant of SIV_{mnd2} interacted with Nup153 despite no interaction observed for WT Vpx. (D) Subcellular localization of wild type and variants of SIV_{rcm} and SIV_{mnd2} Vpx in HeLa cells.

interact with Nup153. Interestingly, SIV_{mnd2} $Vpx^{T59, N61A}$ mutant localized predominantly in the nucleus compared with cytoplasmic localization of WT Vpx (Figure 4D). Collectively, these results suggest that Vpx interaction with Nup153 is evolutionarily conserved and is critical for efficient nuclear translocation of Vpx.

MAPK/ERK-2–dependent Vpx phosphorylation is required for its interaction with Nup153

Results from the coimmunoprecipitation analysis using various Vpx mutants clearly indicate that the serine residues within the 61–80 region of Vpx are critical for its interaction with Nup153. MAPK/ERK-2–mediated phosphorylation of PIC-associated viral proteins was found to be critical in the early steps of virus life cycle (Jacqué *et al.*, 1998; Cartier *et al.*, 1999; Hemonnot *et al.*, 2004). We previously demonstrated that phosphorylation of SIVsm Vpx by virion-associated MAPK/ERK-2 was found to be critical for its efficient nuclear localization (Rajendra Kumar *et al.*, 2005). Therefore, it was intriguing to investigate whether MAPK/ERK-2–mediated Vpx phosphorylation at serine residues orchestrate its interaction with Nup153. Toward this, we first tested the Vpx interactive ability with Nup153 in the presence of MAPK/ERK-2 inhibitors, U0126 and Hypericin. Results in Figure 5A indicate that treatment of GFP–Vpx-expressing HEK293T cells with MAPK inhibitors severely impaired Vpx interaction with Nup153 (top panel, lanes 3 and 4). Further, to understand whether Vpx interaction with Nup153 is dependent on the phosphorylation of serine residues, WT and serine mutant Vpx of SIV_{sm}, SIV_{rcm}, and SIV_{mnd2} were expressed in HEK293T cells and treated with MAPK/ERK-2 inhibitor U0126 prior to coimmunoprecipitation with anti-Nup153 antibodies followed by Western blot with anti-GFP antibodies. Results in Figure 5B clearly indicate that U0126 blocks the interaction of Vpx from SIV_{sm}, SIV_{rcm}, and SIV_{mnd2} ($Vpx^{T59, N61S}$) with Nup153 (top panel, lanes 4, 7, and 10). In addition, U0162 blocked the MAPK/ERK-2 phosphorylation levels without altering its expression (Figure 5B, lanes 4, 7, and 10). Further, to address the specificity of MAPK/ERK-2–mediated phosphorylation of serine residues on Vpx interaction with Nup153, WT and

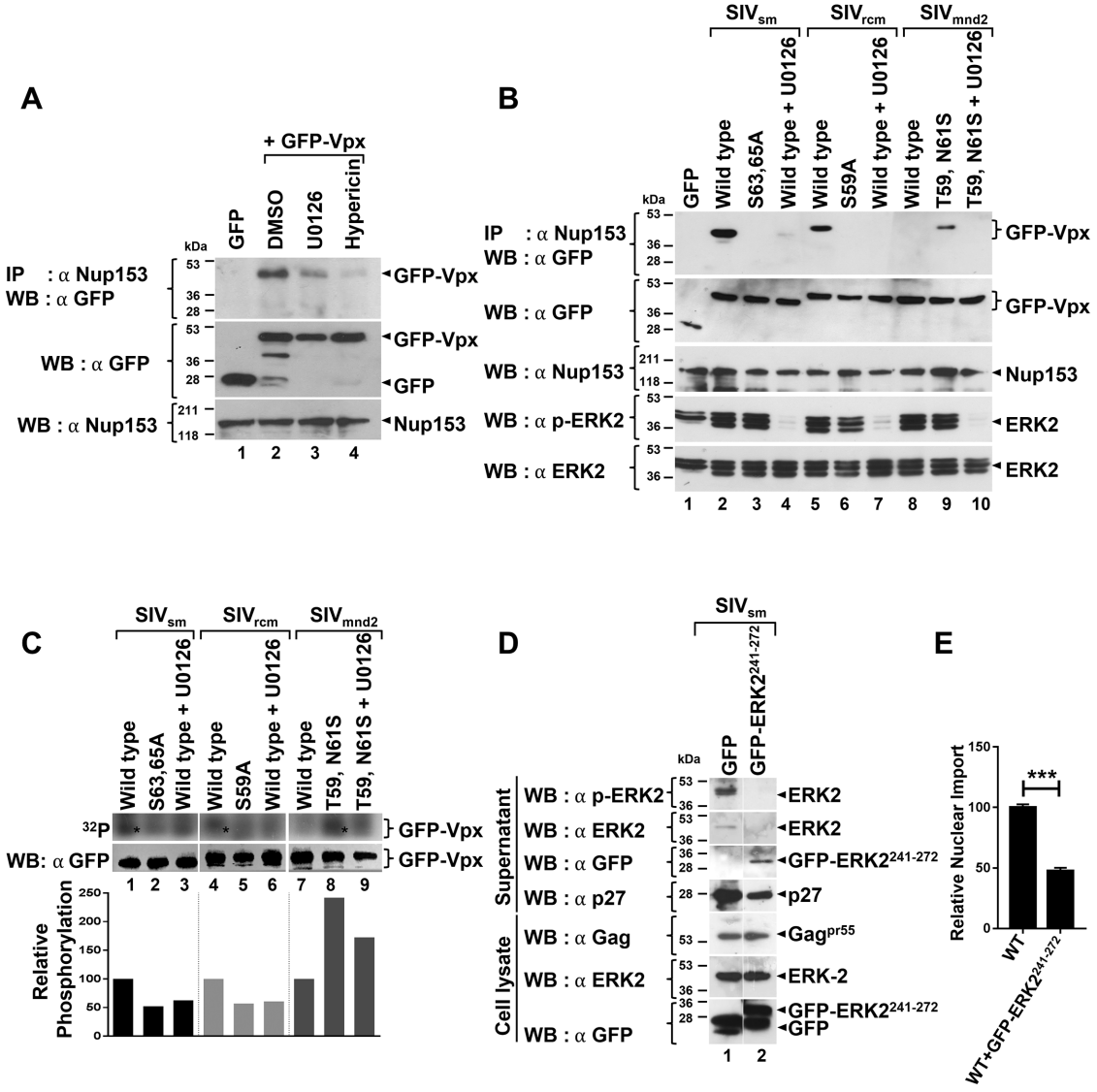

FIGURE 5: MAPK/ERK2-mediated phosphorylation of Vpx is critical for Nup153-dependent nuclear translocation of viral genome. (A) Vpx of SIV_{sm} was expressed in HEK293T cells in the presence of MAPK inhibitors U0126 and Hypericin and the cell lysates were subjected to coimmunoprecipitation with anti-Nup153 antibody followed by Western blot using anti-GFP antibody. Results clearly indicate that inhibition of MAPK activity impairs Vpx interaction with Nup153. (B) Vpx of SIV_{sm}, SIV_{rcm}, and SIV_{mnd2} were ectopically expressed in HEK293T cells in the presence of inhibitor U0126 and the cell lysates were subjected to coimmunoprecipitation with anti-Nup153 antibody followed by Western blot using anti-GFP antibody. (C) Vpx of SIV_{sm}, SIV_{rcm}, and SIV_{mnd2} were ectopically expressed in HEK293T cells in the presence of ^{32}P orthophosphoric acid and the cell lysates were subjected to immunoprecipitation with anti-GFP antibody followed by autoradiography. The asterisks indicate phosphorylation of Vpx variants. Bars in the graph indicate the relative quantitation of Vpx phosphorylation normalized to the WT Vpx expression with respective viral isolate. (D) Virion incorporation of GFP-MAPK/ERK-$2^{241-272}$ was determined by Western blot using anti-GFP antibodies. Results indicate that packaging of endogenous MAPK/ERK-2 was compromised in the presence of GFP-MAPK/ERK-$2^{241-272}$. (E) Quantitative analysis of amplified 2-LTR circles indicates that the nuclear import ability of MAPK/ERK-2 packaging defective virus was severely compromised. Bars in the graph are average mean ± SD from three independent experiments. ***$p < 0.001$ (Student's unpaired *t* test).

indicated serine mutants of Vpx were expressed in HEK293T cells and cultured with ^{32}P orthophosphoric acid containing growth medium with or without U0126. Cell lysates were subjected to immunoprecipitation with anti-GFP antibodies followed by autoradiography as described in *Materials and Methods*. Results in Figure 5C indicate that U0126 significantly impaired MAPK/ERK-2–mediated Vpx phosphorylation without altering their expression (bottom panel, lanes 3, 6, and 9). Exchange of serine residues completely abrogated SIV_{sm} and SIV_{rcm} Vpx phosphorylation (Figure 5C, top panel, lanes 2 and 5). Interestingly, despite no phosphorylation of WT Vpx of SIV_{mnd2}, we observed phosphorylation of mutant $Vpx^{T59, N61S}$ of SIV_{mnd2}, which was reduced in the presence of U0126 (Figure 5C, top panel, lanes 7, 8, and 9). As expected, MAPK inhibitor U0126 abrogated the nuclear translocation of SIVsm Vpx in HeLa cells (Supplemental Figure S5A). In contrast, the nucleolar localization of the nucleolar marker protein, Nucleolin, was not altered in the presence

of U0126 (Supplemental Figure S5B), which suggests the specificity of MAPK/ERK-2–mediated phosphorylation on Vpx nuclear import. Together, these results provided evidence that MAPK/ERK-2–mediated phosphorylation of Vpx is critical for its interaction with Nup153 and the subsequent nuclear translocation.

MAPK/ERK-2–mediated Vpx phosphorylation is critical for efficient nuclear translocation of the SIV genome

Our results clearly suggest that Vpx phosphorylation correlated with its ability to interact with Nup153. We next examined the functional importance of Vpx interaction with Nup153 during the virus life cycle. Reports from others and our laboratories demonstrated that MAPK/ERK-2 was incorporated into the newly formed virions in association with CA region of Gag (p55) polyprotein (Cartier *et al.*, 1997; Jacqué *et al.*, 1998; Hui, 2002; Gupta *et al.*, 2011). Virion-incorporated MAPK/ERK-2 molecules were shown to be important for establishing viral infection in nondividing target cells such as macrophages (Jacqué *et al.*, 1998; Gupta *et al.*, 2011). Considering our observation of MAPK/ERK-2–mediated phosphorylation-dependent interaction of Vpx with Nup153, we next tested the nuclear import efficiency of viral genome in cells infected with MAPK/ERK-2–deficient SIV particles. Toward this, we first identified the minimal domain of MAPK/ERK-2 required for its interaction with Gag polyprotein. Analysis of MAPK/ERK-2 deletion mutants identified the domain between residues 241 and 272 (GFP-ERK-$2^{241\text{-}272}$) was sufficient for its interaction with viral polyprotein Gag and subsequent packaging into new virions (Figure 5D). MAPK/ERK-2–deficient virions were produced by cotransfection of SIV_{sm} proviral DNA with GFP-MAPK/ERK-$2^{241\text{-}272}$ in HEK293T cells. Virus particles were pelleted by ultracentrifugation followed by Western blot to determine the incorporation status of GFP-MAPK/ERK-$2^{241\text{-}272}$ into the virus particles. Results in Figure 5D clearly indicate that coexpression of GFP-MAPK/ERK-$2^{241\text{-}272}$ competes with endogenous MAPK/ERK-2 for its interaction with Gag polyprotein and thereby blocks the incorporation of WT MAPK/ERK-2 into virus particles without altering the expression levels of cellular MAPK/ERK-2 (lane 2). Virions with defective WT MAPK/ERK-2 packaging were used to infect G2/M-arrested LuSIV cells (Roos *et al.*, 2000) as described in *Materials and Methods*. Etoposide was used to induce cell cycle arrest of mammalian cell lines to monitor lentivirus infection by 2-LTR assay (Groschel and Bushman, 2005; Matreyek *et al.*, 2013). The efficiency of Etoposide-mediated G2/M arrest in LuSIV cells was determined by FACS analysis (Supplemental Figure S6A) as described in *Materials and Methods*. Genomic DNA was isolated from virus-infected LuSIV cells to amplify the U3/U5 junction region (Supplemental Figure S6B) of the 2-LTR circle (present exclusively in the nucleus), a hallmark of viral genome nuclear import. A significant decrease in 2-LTR circle formation was observed in the cells infected with MAPK/ERK-2–deficient SIV particles compared with cells infected with virions containing full-length MAPK/ERK-2 (Supplemental Figure S6C, lanes 1 and 2, and Figure 5E). Collectively, these results provided evidence that MAPK/ERK-2–mediated phosphorylation promotes Vpx interaction with human Nup153, which in turn facilitates the efficient nuclear translocation of the SIV genome for the successful establishment of virus infection in nondividing target cells.

DISCUSSION

Lentiviruses have evolved strategies to translocate their genome across the intact nuclear envelope of nondividing target cells for successful integration into the host cell chromosome. Interestingly, multiple viral proteins have been implicated for the efficient transport of the HIV-1 genome into the nucleus of nondividing target cells (Bukrinsky *et al.*, 1993; Heinzinger *et al.*, 1994; Mahalingam *et al.*, 1997; Haffar *et al.*, 2000; Dvorin *et al.*, 2002; Ikeda *et al.*, 2004; Yamashita *et al.*, 2007; Schaller *et al.*, 2011). However, Vpx was found to be an indispensable candidate for nuclear import of the HIV-2/SIV genome since virions containing truncated Vpx were found to be severely defective for productive infection of terminally differentiated cells (Hirsch *et al.*, 1998; Mahalingam *et al.*, 2001; Belshan *et al.*, 2006). Reports from others and our laboratories showed that Vpx contains multiple NLS sequences and predominantly localized in the nuclear/perinuclear region, notably, NLS at c-terminus transports cargo into the nucleus by a novel noncanonical pathway (Pancio *et al.*, 2000; Mahalingam *et al.*, 2001; Rajendra Kumar *et al.*, 2003; Belshan *et al.*, 2006; Singhal *et al.*, 2006a). Furthermore, wheat germ agglutinin effectively inhibited the Vpx nuclear translocation (Singhal *et al.*, 2006a) indicating a role for NPC proteins on Vpx nuclear translocation. Collectively, these reports suggest that Vpx enters into the nucleus by multiple mechanisms and one such mechanism could be by its interaction with the components of NPC. Genomewide siRNA screening suggested that Nup153 is one of the critical factors for efficient HIV-1 replication (Ebina *et al.*, 2004; Brass *et al.*, 2008; König *et al.*, 2008; Zhou *et al.*, 2008). Recent reports from others and our laboratories demonstrated that human Nup153 interacts with HIV-1 Vpr, IN, and CA (Varadarajan *et al.*, 2005; Woodward *et al.*, 2009; Matreyek and Engelman, 2011) to translocate the viral genome into the nucleus of nondividing target cells. Despite the fact that Vpx is the major determinant for the nuclear transport of the HIV-2/SIV genome in nondividing cells, whether it interacts with components of NPC remains unknown.

In the present investigation, we have provided evidence that a physical interaction between HIV-2/SIV Vpx and human Nup153 is critical in facilitating the contact between the subviral particles and host cell NPC. Coimmunoprecipitation experiments using Vpx variants suggest that amino acids between positions 61 and 80 of Vpx are critical for its interaction with Nup153. Combination of deletion mutagenesis and coimmunoprecipitation analysis reveals that the zinc-finger domain of Nup153 is critical for its interaction with Vpx. Zinc-finger domain of Nup153 was involved in importin receptor-independent nuclear entry of a subset of cargo proteins (Liu *et al.*, 2003; Higa *et al.*, 2007), suggesting that Nup153 may help in docking of the viral genome at NPC. Interaction of Nup153 with Vpx from different SIV isolates and HIV-2 suggests that this interaction is evolutionarily conserved among primate lentiviruses. Further, the exclusive cytoplasmic localization Nup153 interaction-defective SIV_{sm} $Vpx^{S63A,65A}$ suggests that serine residues are critical for Vpx interaction with Nup153 and its nuclear localization. This was further supported by the cytoplasmic localization of SIV_{rcm} Vpx^{S59A} mutant as well as the WT SIV_{mnd2} Vpx. Interestingly, replacement of WT T59 and N61 with serines in SIV_{mnd2} Vpx facilitated the mutant protein interaction with Nup153 and localized to the nuclear (predominant) with a diffused cytoplasmic pattern that suggests a "gain of function" for the Vpx mutant. Furthermore, depletion of endogenous Nup153 severely impaired Vpx translocation into the nucleus. Unlike most cellular proteins, lentiviral proteins are known to encode canonical as well as noncanonical NLS sequences, suggesting that multiple mechanism(s) are involved in the nuclear translocation of viral genome. Interestingly, studies with knockdown of several nucleoporins provided evidence that interaction of viral proteins with nucleoporins via noncanonical NLS sequences might be critical for efficient nuclear import of viral genome in a cell division-independent manner (König *et al.*, 2008; Kane *et al.*, 2018).

These findings further support our observation on the cytoplasmic localization of Vpx in Nup153-depleted cycling HeLa cells. Comprehensive analysis of results from 3D-SIM and live cell imaging of Vpx-expressing HeLa cells suggest that Nup153 is essential for Vpx nuclear translocation. It is known that Vpx encodes multiple NLS sequences and are critical for its efficient nuclear localization (Pancio *et al.*, 2000; Mahalingam *et al.*, 2001; Rajendra Kumar *et al.*, 2003; Belshan *et al.*, 2006). The observed diffused nuclear localization pattern of $SIV_{mnd2}^{T59,N61S}$ might be due to the differential functional ability of its amino-terminal NLS, which requires further experiments. Together, these data provided evidence that the conserved serine residues in Vpx are critical for its interaction with human Nup153 and efficient nuclear translocation.

Phosphorylation of viral proteins was found to be critical for early events in the virus life cycle (Francis *et al.*, 2011). The current investigation demonstrates that MAPK/ERK-2–mediated Vpx phosphorylation was found to be critical for its interaction with human Nup153. Furthermore, metabolic labeling of WT and mutant Vpx proteins in the presence of MAPK inhibitors confirmed that Vpx was phosphorylated at conserved serine residues by MAPK/ERK-2. Consistent with the previous report on Vpx phosphorylation (Rajendra Kumar *et al.*, 2005), we found that nuclear localization of Vpx was severely impaired in the presence of MAPK inhibitors, which suggests that phosphorylation of Vpx may be critical in promoting/stabilizing its interaction with Nup153. Recent reports suggest that the depletion of Nup153 results in genome instability, alters cell survival, and promotes defects in early mitosis with the accumulation of multilobed nuclei (Draviam *et al.*, 2007; Mackay *et al.*, 2009; Moudry *et al.*, 2012), despite that it was shown to be critical for HIV-1 infectivity in nondividing host cells (Brass *et al.*, 2008; König *et al.*, 2008; Zhou *et al.*, 2008). Since the Nup153 depletion leads to the alteration of cell morphology and that MAPK/ERK-2–mediated Vpx phosphorylation is critical for its interaction with Nup153 and nuclear localization, we evaluated the functional relevance of MAPK/ERK-2–mediated Vpx phosphorylation on the nuclear import of the viral genome in growth-arrested cells. The observed reduction of viral genome nuclear transport (less 2-LTR circles) in MAPK/ERK-2–deficient SIVsm-infected growth-arrested cells provided evidence that MAPK/ERK-2–mediated Vpx phosphorylation promotes its interaction with Nup153 and facilitates efficient nuclear translocation of the viral genome in nondividing target cells.

It is known that viral proteins, namely Vpr, IN, and CA, are involved in the nuclear transport of HIV-1 PIC (viral genome). Interestingly, these viral proteins encode multiple NLSs (canonical as well as noncanonical) and interact with more than one transport receptor (importins, transportin, and nucleoporins) for translocating HIV-1 PIC into the nucleus in nondividing target cells (Varadarajan *et al.*, 2005; Woodward *et al.*, 2009; Krishnan *et al.*, 2010; Matreyek and Engelman, 2011; Di Nunzio *et al.*, 2013; Matreyek *et al.*, 2013). HIV-1 CA interaction with Nup153 has been shown to be critical for nuclear import of viral PIC (Di Nunzio *et al.*, 2013; Matreyek *et al.*, 2013). On the other hand, in the case of HIV-2/SIV, Vpx but not Vpr is the major determinant for the nuclear transport of viral genome in nondividing target cells. Furthermore, SIVsm with defective Vpr replicate as like WT virus in macaque macrophage cultures (Fletcher *et al.*, 1996; Hirsch *et al.*, 1998; Mahalingam *et al.*, 2001). In the current investigation, we have not ruled out the possibility of SIV CA interaction with human Nup153, which warranted further experiments. Recent reports suggest that Vpx promotes proteasomal degradation of host cell restriction factor SAMHD1, a triphosphohydrolase, which is responsible for depleting the cytoplasmic dNTPs pool required for viral DNA synthesis in nondividing cells (Hrecka *et al.*, 2011; Laguette *et al.*, 2011). Interestingly, Vpx interacts with SAMHD1 via its amino terminal domain (Ahn *et al.*, 2012; Hofmann *et al.*, 2012); in contrast, human Nup153 interacts with the carboxyl domain (amino acids 61–80) of Vpx, suggesting that Vpx encodes distinct binding domains for Nup153 and SAMHD1 and are nonoverlapping. Together, these results provided evidence that SAMHD1 degradation activity of Vpx is independent of its interaction with Nup153. Furthermore, Vpx-mediated SAMHD1 degradation alone may not be sufficient for efficient lentiviral infection (Fujita *et al.*, 2012; Li *et al.*, 2015; Baldauf *et al.*, 2017). Nevertheless, it would be interesting to understand whether Vpx-mediated proteasomal degradation of SAMHD1 has any indirect effect on cellular trafficking of the viral genome across NPC. Collectively, we propose a model (Figure 6) that MAPK/ERK-2–mediated phosphorylation of HIV-2/SIV Vpx is critical for its interaction with human Nup153 for efficient nuclear transport of lentiviral genome to establish viral infection in nondividing target cells.

MATERIALS AND METHODS

Expression plasmids and $SIV_{smPBj1.9}$ provirus constructs

Vpx of $SIV_{smPBj1.9}$ (Accession number: M31325.1), $HIV\text{-}2_{ISY}$ (Accession number: J04498.1), $SIV_{rcmGAB1}$ (Accession number: AF382829.1), and $SIV_{mnd14cg}$ (Accession number: AF328295.1) were cloned as N-terminal GFP or Flag tag fusion in pCDNA3 (Invitrogen Life Technologies, USA). Vpr of $SIV_{smPBj1.9}$ was PCR amplified and cloned as C-terminal GFP fusion in pCDNA3. Vpx deletions (1–63, 1–40, 61–80, and 64–112) and site-specific mutants (W49,53,56S; S63,65A; S59A and T59, N61S) were generated as N-terminal GFP or Flag fusion in pCDNA3. The full-length Nup153 was expressed as GFP fusion in the pEGFP-N2 vector. $MAPK/ERK2^{241\text{-}272}$ expression construct was generated as N-terminal GFP fusion in pCDNA3. Gene-specific primers were used to amplify each of Vpr/Vpx variants. Site-specific mutants of $SIV_{smPBj1.9}$ Vpx proviral clones were generated without altering the overlapping Vif open reading frame as described previously (Rajendra Kumar *et al.*, 2003).

Cell culture, transfection, immunoprecipitation, and Western blot analysis

HEK293T and HeLa cells (NIH AIDS Reagent Program) were maintained in DMEM (Invitrogen Life Technologies, USA) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (Invitrogen Life Technologies, USA). LuSIV cells (NIH AIDS Reagent Program) were maintained in RPMI 1640 (Invitrogen Life Technologies, USA) supplemented with 10% FBS, 1% antibiotic-antimycotic (Invitrogen Life Technologies, USA), and 300 µg/ml hygromycin B (Sigma, USA). All cell lines used in the current investigation were received from the NIH AIDS Research Reagent Program and certified that cell lines are free of mycoplasma contamination. We have frequently checked the morphology of the cells by microscopy for authentication.

For efficient protein expression, cells were infected with vTF7-3, a recombinant vaccinia virus expressing T7 RNA polymerase, for 1 h, followed by transfection using lipofectamine (Invitrogen Life Technologies, USA) according to manufacturer's protocol. Cells were harvested and lysed in 1× cell lysis buffer (25 mM Tris-HCl, pH 7.4, 150 mM KCl, 1 mM Na_2EDTA, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid [EGTA], 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 0.4 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM NaF, 1 mM Na_3VO_4, and 1 µg/ml each of aprotinin, leupeptin, and pepstatin). For immunoprecipitation of endogenous Nup153, lysates were incubated with rabbit polyclonal

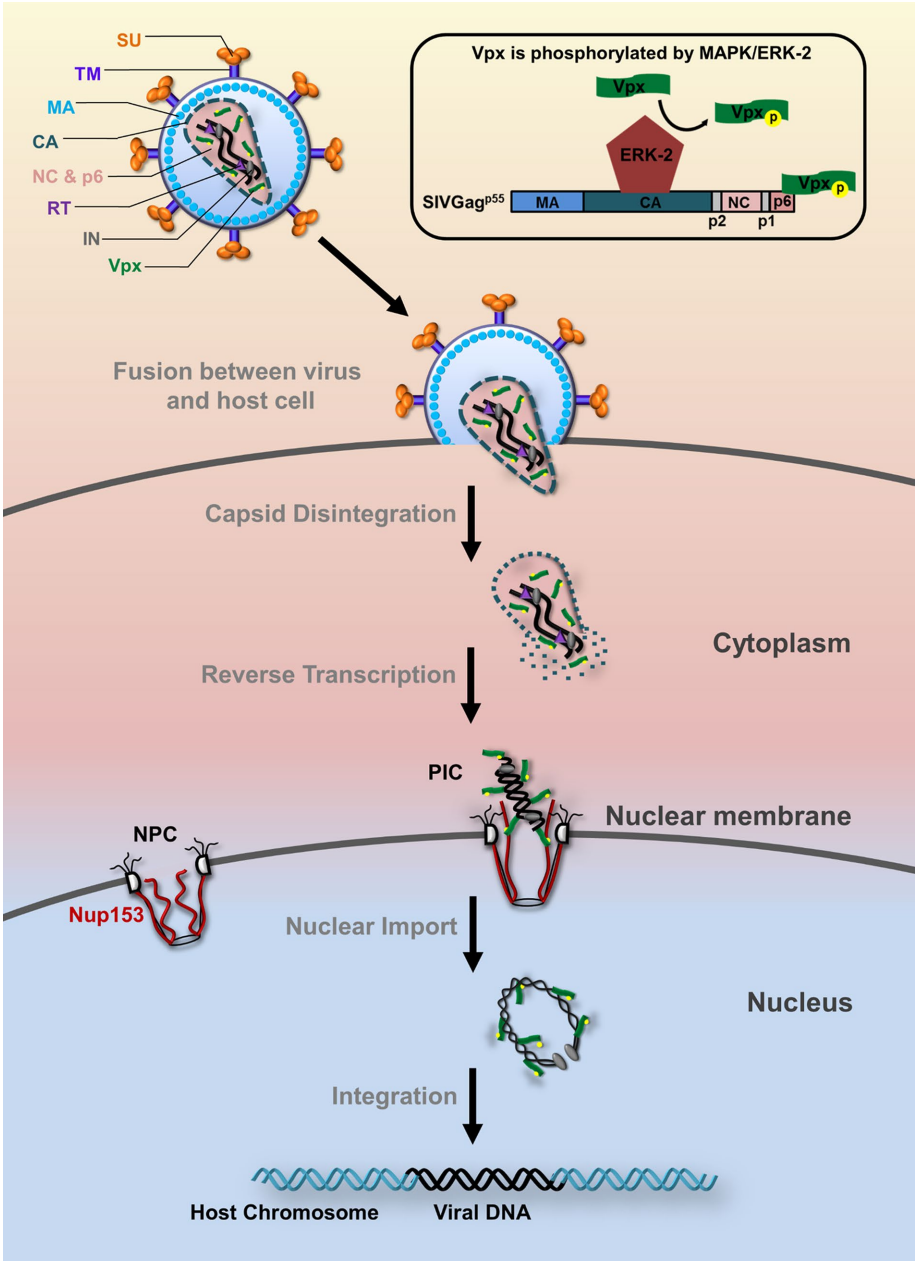

FIGURE 6: Proposed model for the mechanism of Vpx-mediated nuclear import of HIV-2/SIV genome. Host cell MAPK/ERK-2 phosphorylates Vpx. The fusion between virus and host cell membrane resulted in the release of viral core into the host cell cytoplasm. The gradual disintegration of CA molecules and synthesis of viral DNA resulted in the formation of the viral PIC. Interaction of PIC-associated Vpx with Nup153 at NPC promotes the nuclear translocation of the viral genome. SU, envelope surface unit; TM, envelope transmembrane protein; MA, matrix; CA, capsid; NC, nucleocapsid; RT, reverse transcriptase; IN, integrase; NPC, nuclear pore complex.

anti-Nup153 antibody (cat #Ab84872, 1:500 dilution, Abcam, UK). Coimmunoprecipitation of Nup153 deletions was performed by incubating lysates with mouse monoclonal anti-HA antibody (cat #Sc-7392, 1:1000 dilution, Santa Cruz Biotechnology, USA). Protein A sepharose-bound protein complexes were resolved on SDS–12% PAGE and transferred to Hybond-P membrane (GE Healthcare, Sweden) followed by Western blot with the monoclonal anti-GFP antibody (B-2, cat #Sc-9996, 1:1000 dilution, Santa Cruz Biotechnology, USA) for GFP-Vpx. The protein–antibody complexes were probed using horseradish peroxidase–conjugated anti-mouse (cat #1010-05,1:3000 dilution, Southern Biotechnology, USA) or anti-rabbit antibodies (cat #4010-05, 1:3000 dilution, Southern Biotechnology, USA) and detected using the Enhanced Chemiluminescence Prime detection system (GE Healthcare, Sweden). All experiments were repeated three times.

Immunostaining and confocal microscopy

Recombinant vaccinia virus (vTF7-3)-infected HeLa cells cultured on chamber culture slides (BD Biosciences, USA) were transfected with WT or mutant Vpx expression plasmids. After 1620 h, cells were fixed with 4% (wt/vol) paraformaldehyde followed by permeabilization using 0.1% Triton X-100. Endogenous Nup153 was depleted with Nup153-specific shRNA (cat #TLHSU1400-9972, TransOMIC Technologies, USA) for 72 h. To visualize endogenous Nup153, cells were incubated with the anti-Nup153 antibody (cat #Ab84872, 1:250 dilution, Abcam, UK) for 90 min at 37°C and probed with Alexa Fluor 594–conjugated anti-rabbit immunoglobulin G (1:1000 dilution, Molecular Probes, USA). Lamin A/C and NPC were visualized using anti-Lamin A/C (cat #Sc-7292, 1:250 dilution, Cell Signaling Technology, USA) and anti-NPC antibodies (cat #N8786, 1:250 dilution, Sigma, USA), respectively. The cells were mounted in mounting medium (Vector Laboratories, USA) containing DAPI to stain cell nuclei. Cells were imaged with LSM710 laser scanning confocal microscope (Carl Zeiss, Germany) using ZEN 2009 software (Carl Zeiss, Germany). All experiments were repeated three times.

SR-SIM

SR-SIM images were acquired with Elyra PS.1 Super Resolution microscopy (Carl Zeiss, Germany) using a Plan-Apochromat 63×/1.4 Oil (NA of 1.4), 1.6× tube lens with a collar for correction according to coverslip thickness, and immersion medium (Immersol 518F with a refractive index of 1.518). Laser lines at 405, 488, 561, and 647 nm were used for excitation. SIM images (five different angular orientations of illumination for each SIM image) were captured with a cMOS camera (pco.edge 5.5, 2560 × 2160 pixels at 6.5 µm pixel size; 16-bit dynamic range, peak quantum efficiency >60% or equivalent and field of view ~80 × 80 µm^2).

Multicolor 3D-SIM image acquisition

Each image was acquired with an exposure time of 80 and 100 ms. Five orientation angles of the excitation grid were acquired for each Z-plane with Z-spacing of 110 nm between planes. SIM images were processed with the SIM module using ZEN BLACK software (Carl Zeiss, Germany). Representative SIM images were analyzed

using ZEN Blue software for 3D-Model reconstruction with a 180-frame rotation series along the Y-axis and exported as AVI videos. The total thickness of the sample was about 4 to 5 µm, leading to the acquisition of 25–30 slices. The absolute intensity values between the wide field and SIM images were normalized using OriginPro to a range between 0 and 1 and plotted against distance (µm) (Supplemental Figure S2A). The line profiles were drawn perpendicular to the nuclear membrane acquired by SIM based on visual inspection of the thickness or the distribution of the NPC that corresponds to Nup153 expression.

Live cell imaging

Cell imaging dishes (Eppendorf, Germany) containing HeLa cells were transfected with GFP-Vpx expression plasmids and maintained in an environmentally controlled chamber attached with a fluorescence microscope (Carl Zeiss, Germany). Images were acquired at 2-min intervals using Plan-Apochromat 20× objective (NA-0.8) and a CCD camera (AxioCam HRm, Carl Zeiss, Germany). Images were analyzed using ZEN Blue software and exported as AVI videos. All experiments were repeated three times.

Metabolic labeling

HEK293T cells were grown on 60-mm plates and transiently transfected with WT or mutant Vpx constructs. For metabolic labeling, 12 h posttransfection, cells were incubated with 1.5 ml of phosphate-free DMEM containing 1% dialyzed FBS and 1 mCi/ml [^{32}P] orthophosphate (Bhabha Atomic Research Center, India) for 6 h. Cells were treated with MAPK inhibitor, U0126 for 30 min, and lysed with radioimmunoprecipitation buffer containing 1% (vol/vol) Triton X-100, 0.5% (wt/vol) deoxycholate, 0.2 mM PMSF, and 0.2 mM Na_2VO_4. Cell lysates were incubated with anti-GFP antibody for 2 h followed by 90-min incubation with protein A sepharose beads with gentle rotation at 4°C. Immunoprecipitates were separated using the SDS–12% PAGE, transferred to Hybond-P membrane (Amersham Biosciences, Sweden), and exposed to XAR-5 film (Eastman Kodak, USA) at –80°C.

Viruses and virus-like particles (VLPs)

Virus stocks were generated by transfecting HEK293T cells with WT $SIV_{smPBj1.9}$ proviral clones using Lipofectamine-2000 (Invitrogen Life Technologies, USA). Virus-like particles were generated by transfecting HEK293T cells with Gag expression plasmid. To produce viruses or VLPs defective for MAPK/ERK-2 packaging, WT $SIV_{smPBj1.9}$ proviral plasmids were cotransfected with GFP-MAPK/ERK-$2^{241-272}$ expression vector. For Western blot analysis, the culture supernatant was collected and concentrated by centrifugation through Amicon 30 cut-off concentrators. Pellets from concentrated virus or virus-like-particles were resolved on SDS–10% PAGE and probed with anti-Gag or anti-GFP antibodies.

Virus infection and 2-LTR assay

LuSIV cells (NIH AIDS Reagent Program; cat #5460; a reporter cell line derived from CEMx174 cells) were infected with WT or MAPK/ERK2 packaging-deficient SIV_{sm} and analyzed for the nuclear import efficiency of the viral genome. Briefly, LuSIV cells were incubated with 1 µM etoposide for 24 h, and cell cycle arrest at the G2/M phase was confirmed by FACS analysis. G2/M-arrested LuSIV cells (1×10^6) were infected with $SIV_{smPBj1.9}$ virions (100 ng equivalent $p27^{gag}$) containing full-length MAPK/ERK2 or GFP-MAPK/ERK2$^{241-271}$. Cells were harvested 6 h postinfection, genomic DNA was isolated, and 200 ng of genomic DNA were used as a template to PCR amplify U3/U5 junction (318 base pairs) using 2-LTR-U5+ (5′-GCCAGTGTGTGTTCCCATCTCTCCTAGTCG-3′) as forward and 2-LTR-U3– (5′-AGAGCGAAATGCAGTTGTATTTATACAGAG-3′) as reverse primer. The region of Gag p27 was amplified as a control for the quantitation of total viral DNA synthesis, and 2-LTR levels were normalized with total viral DNA synthesis.

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