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The Functionally Conserved Nucleoporins Nup124p from Fission Yeast and the Human Nup153 Mediate Nuclear Import and Activity of the Tf1 Retrotransposon and HIV-1 Vpr[V]

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We report that the fission yeast nucleoporin Nup124p is required for the nuclear import of both, retrotransposon Tf1-Gag as well as the retroviral HIV-1 Vpr. Failure to import Tf1-Gag into the nucleus in a *nup124* null mutant resulted in complete loss of Tf1 transposition. Similarly, nuclear import of HIV-1 Vpr was impaired in *nup124* null mutant strains and cells became resistant to Vpr's cell-killing activity. On the basis of protein domain similarity, the human nucleoporin Nup153 was identified as a putative homolog of Nup124p. We demonstrate that in vitro–translated Nup124p and Nup153 coimmunoprecipitate Tf1-Gag or HIV-1 Vpr. Though full-length Nup153 was unable to complement the Tf1 transposition defect in a *nup124* null mutant, we provide evidence that both nucleoporins share a unique N-terminal domain, Nup124p$^{\Delta A264-454}$ and Nup153$^{\Delta A448-634}$ that is absolutely essential for Tf1 transposition. Epigenetic overexpression of this domain in a wild-type (*nup124*$^{+}$) background blocked Tf1 activity implying that sequences from Nup124p and the human Nup153 challenged the same pathway affecting Tf1 transposition. Our results establish a unique relationship between two analogous nucleoporins Nup124p and Nup153 wherein the function of a common domain in retrotransposition is conserved.

INTRODUCTION

Long terminal repeat (LTR)-containing transposons are a diverse group of retroelements that inhabit a variety of eukaryotic hosts. Tf1 is an active LTR-retrotransposon that propagates within the genome of the fission yeast, *Schizosaccharomyces pombe*. Tf1 has coding sequences for Gag, protease (PR), reverse transcriptase (RT), and integrase (IN) proteins and its propagation in fission yeast is known to require many of the same processes used by retroviruses to complete its life cycle within the host cell (Levin *et al.*, 1993). The full-length transcript of the Tf1 retroelement consisting of Gag, PR, RT, and IN proteins assemble along with copies of RNA to form virus-like particles (VLP) or preintegration complexes (PIC) that contain a 26-fold molar excess of Gag relative to IN (Atwood *et al.*, 1996). The presence of Gag is essential for the formation of VLPs and reverse transcription of the RNA into double-stranded cDNA (Teysset *et al.*, 2003). To integrate its cDNA into the host genome, the Tf1 VLP/PIC containing the IN and cDNA must be imported into the nucleus (Dang and Levin, 2000).

HIV-1 Vpr is a 14-kDa, 96 amino acid virion-associated protein that is highly conserved among primate lentiviruses HIV-1, HIV-2, and the simian immunodeficiency virus (SIV; Bukrinsky and Haffar, 1997; Sherman *et al.*, 2002a; Tungaturthi *et al.*, 2003). Unlike the accessory proteins Vif and Nef, incorporation of Vpr has been shown to be specific, involving a distinct domain, the p6 region in HIV-1 Gag and thus, its relative amount within the virion may be closely linked with that of Gag (Lu *et al.*, 1993, 1995; Selig *et al.*, 1999; Tungaturthi *et al.*, 2003). Vpr has been implicated as one of the significant agents in AIDS pathogenesis (Sherman *et al.*, 2002b; Tungaturthi *et al.*, 2003) although the molecular mechanisms underlying such claims are far from understood.

In a manner analogous to HIV-1 Vpr requirement for nuclear import of its PIC (Lu *et al.*, 1993; Mahalingam *et al.*, 1995b, 1997a, 1997b; Fouchier *et al.*, 1998; Popov *et al.*, 1998a, 1998b; Fouchier and Malim, 1999; Le Rouzic *et al.*, 2002), the transposition efficiency of the Tf1-retrotransposon correlated with its ability to import TF1-Gag into the nucleus (Balasundaram *et al.*, 1999; Dang and Levin, 2000). Though the Tf1 retrotransposon lacks the accessory protein Vpr, its Gag protein plays a critical role in the packaging and nuclear import of its VLP resulting in elevated transposition levels

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(Levin *et al.*, 1993; Atwood *et al.*, 1996; Dang and Levin, 2000; Teysset *et al.*, 2003). HIV-1 Vpr and Tf1-Gag proteins (a) display evident karyophilic properties and localize to the nucleus although they do not contain any canonical NLS (b) are required for the nuclear import of the genome of retrotransposon Tf1 (Gag) and HIV-1 (Vpr), (c) form an integral part of VLP in the case of Tf1 Gag and HIV-1 preintegration complex in the case of Vpr, and (d) are essential for optimal HIV-1 replication in macrophages (Vpr) and transposition of Tf1 (Gag; Fouchier *et al.*, 1998; Fouchier and Malim, 1999; Dang and Levin, 2000; Teysset *et al.*, 2003).

Given the importance of nuclear transport and the NPC in the etiology of human disease (Hutchison, 2002; Cronshaw and Matunis, 2004; Kau *et al.*, 2004), the question of how retroviral elements transcend this physical barrier of the nuclear membrane is both an interesting and an important one to consider as a possible means of blocking viral replication. We had previously reported that a nonessential nucleoporin Nup124p of *S. pombe*, was essential for Tf1 transposition (Balasundaram *et al.*, 1999). Here, we have used retrotransposition in the fission yeast as a genetic tool and the involvement of Nup124p to understand the mechanism underlying nuclear transport of Tf1-Gag and the HIV-1 accessory protein Vpr.

MATERIALS AND METHODS

Yeast Strains and Media

All strains are listed in Table 1. The *S. pombe* minimal liquid and plate media were composed of EMM (Balasundaram *et al.*, 1999). Strain YNB19, *h− ura4-294 leu 1-32 Δhis3 Δnup124::HIS3⁺ ade6-M216* and YNB16, *h− leu1-32 ura4-294* were the principal yeast strains used in this study and referred to in the text as null mutant or Δ*nup124* and wild-type or *WT*, respectively. For some experiments YNB38 was used, *ade6-M216, leu1-32, his3-D1, ura4-D18 h−*. Although the construction of strain YNB19 (YHL7143) was described earlier (Balasundaram *et al.*, 1999), experiments using the same were not elaborated till this report. YNB19 and YNB16 were transformed with various plasmids (Tables 1 and 2). Transformants were propagated in EMM −ura − leu + 15 μM thiamine routinely. When expression of the desired sequence was required, strains were grown in EMM −ura/+/−leu without thiamine to induce the *nmt1* or *nmt81* promoter.

Plasmid Constructions

DNA fragments used to create plasmids for this study (Table 2) were generated by PCR using high-fidelity enzyme Turbo Pfu (Stratagene, La Jolla, CA). Oligonucleotide primers are listed in Table 3. All constructs were confirmed by DNA sequencing. huNup153 in pcDNA1 (BNB405, Table 2) was a generous gift from Michael J. Matunis, Johns Hopkins University. pCDL280 and pCDL28 (Table 2) were a generous gift from Mohan Balasubramanian, Temasek Life Sciences Laboratory.

Molecular and Genetic Techniques

Tf1 transposition and Tf1 cDNA recombination were assayed as previously described (Balasundaram *et al.*, 1999). Briefly, Tf1 transposition was monitored by placing a *neo*-marked Tf1 element under the control of an inducible *nmt1* promoter. The bacterial *neo* gene allowed cells to grow in the presence of 500 μg of G418/ml. Tf1 cDNA in the nucleus was examined by cDNA recombination assays and is correlated with the ability of cells to grow on G418-containing medium. Tf1 transposition activity was assayed in strains with the *neoAI*-marked Tf1 plasmid that were induced for the expression of Tf1 on EMM −Ura plates without thiamine for 4 d at 32°C. The plates were then replica printed to EMM plates containing 1 mg of 5-fluoroorotic acid (FOA)/ml. Recombination between cDNA and cellular transposon sequences was scored on FOA-G418 plates after 38 h of growth at 32°C.

Immunofluorescence Microscopy

The nuclear envelope was stained using MAb414 antibodies (Covance, Berkley, CA) that recognize FXFG-repeats in nucleoporins (Davis and Blobel, 1986). HA-tagged proteins were visualized with antihemagglutinin antibody (clone 12CA5, Roche Biochemicals, Basel, Switzerland). Cells were harvested at 0.4–0.8 OD_{600} and treated for indirect immunofluorescence as previously described (Balasundaram *et al.*, 1999) with the primary antibody anti-HA at a dilution of 1:1000 or MAb414 at 1:200 dilution. Either Alexa Fluor 488 or Alexa Fluor 594 (Molecular Probes, Eugene, OR) was used as the secondary antibody at a 1:1000 dilution and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). All epi-fluorescence microscopy were performed at 1000× magnification. A Leica (Deerfield, IL) DMLB microscope equipped with an Optronics DEI-750T coded CCD camera with Leica Qwin proprietary software was used to capture images that were processed for figure presentation using Adobe Photoshop 5.0 software. Laser-scanning confocal microscopy was performed using a Zeiss LSM 510 confocal microscope equipped with a krypton/argon laser as the light source. Images were captured for CFP (excitation at 458 nm at 11% and emission at 475–500 nm), GFP (excitation at 488 nm at 11% and collection of the signal from 505 nm), YFP (excitation at 488 nm at 11% and emission at 610–640 nm) and DAPI (excitation at 780 nm at 100% and collection of the signal from 435–485 nm). Images were processed using Metamorph software (Universal Imaging, West Chester, PA).

Coimmunoprecipitation and In Vitro Binding Assay

Nup124, TF1-Gag:FLAG, HIV-1 Vpr:FLAG, SIV Vpr:FLAG, SIV Gag:FLAG, Nup153, and *GST:6His:S.tag:Kap95* from BNB401, BNB349, BNB422, BNB430, BNB429, BNB405, and BNB400, respectively (Table 2), were transcribed and translated in the presence of [^{35}S]methionine using a TNT-coupled Reticulocyte Lysate System (Promega, Madison, WI) according to the manufacturer's instructions. Equimolar amounts of in vitro–translated proteins were mixed and incubated for 60 min in a binding buffer containing 25 mM HEPES (pH 7.9), 150 mM KCl, 0.1%, Nonidet P-40, 5% glycerol, 0.5 mM dithiothreitol, and 0.4 mM phenylmethylsulfonyl fluoride. Respective antibodies were added to each tube with 400 μl of binding buffer and incubated for 90 min. Protein G-Sepharose (5 mg per tube) was added to all the tubes, which were then incubated for 90 min on a nutator. The beads were washed three times with the binding buffer. The immunoprecipitated protein complexes were eluted from the Sepharose beads and subjected to SDS/PAGE in 8–16% gels. The gels were processed for fluorography. All incubations were carried out at 4°C unless otherwise noted.

RESULTS

Nuclear Translocation of Tf1-Gag Requires Nup124p

We had previously reported that Nup124p, a nuclear pore complex factor was absolutely required for Tf1 transposition and that elimination of Tf1 transposition activity in a *nup124-1* mutant was associated with the inability of Tf1-Gag to be localized in the nucleus (Balasundaram *et al.*, 1999). To differentiate whether this behavior resulted from lack of recruitment of the Tf1-Gag to the nuclear envelope or translocation of Tf1-Gag into the nucleus in a Δ*nup124* mutant, we expressed an inducible YFP-tagged Tf1-Gag construct in *Wild-type* (*WT*) and Δ*nup124* host strains. The nuclear envelope was stained with MAb414 that specifically reacts with FXFG-containing nucleoporins at the nuclear envelope (Davis and Blobel, 1986) and nuclear DNA was stained with DAPI. As depicted in Figure 1, both strains exhibited aggregates in the cytoplasm, a feature that is suggestive of the presence of VLPs (Teysset *et al.*, 2003). The *WT* strain exhibited YFP-Gag fluorescence *within* the nucleus, colocalizing with the DAPI stain. However, in contrast, the Δ*nup124* strain exhibited a perinuclear staining of YFP-Gag at the nuclear envelope (NE) that colocalized with the MAb414 staining corresponding to FXFG-nucleoporins. No YFP-Gag fluorescence was visible within the nucleus. These observations were confirmed by confocal-laser scanning *Z*-series imaging (unpublished data). We may thus conclude that Tf1 is recruited to the nuclear envelope even in the absence of Nup124p but is unable to be translocated *into* the nucleus as is depicted for the wild-type.

Domains of Nup124p Required for Its Function

As deduced from its amino acid sequence, Nup124p contains two major domains. An N-terminal domain containing five clusters of basic amino acids and a C-terminal domain containing eleven FXFG repeats (Figure 2A). To determine the functionality of these domains with respect to activity of Tf1-Gag as well as nuclear localization, we deleted the abovementioned domains. Mutant *nup124* constructs were

Table 1. Yeast strains

Strain (YNB)	Parent strain/source	Plasmid				Source or reference
		ura$^+$		*Leu*$^+$		
		Number	Character	Number	Character	
16	YHL912	—		—		Balasundaram *et al.* (1999)
19	YHL7143	—		—		Balasundaram *et al.* (1999)
7	YNB19	pHL449-1	Tf1 reporter: *neoAI*-marked version of *nmt1: Tf1* in an *ura4* selectable plasmid	pHL1339–2	*Nup124* transcribed from its native promoter	This study
8	YNB19	pHL449-1	Tf1-reporter (see above)	pSP1	Empty vector	This study
10	YHL4990	pHL490	*PROT*^{fs} *(Frameshift mutation in Tf1-protease)*	pSP1	Empty vector	Balasundaram *et al.* (1999)
11	YHL4992	pHL476	*IN*^{fs} *(Frameshift mutation in Tf1-integrase)*	pSP1	Empty vector	Balasundaram *et al.* (1999)
58	YNB19	pHL449-1	Tf1-reporter (see above)	pBNB12	*3HA:Nup124*	This study
244	YNB19	pHL449-1	Tf1-reporter (see above)	pBNB119	*nup124* $^{\Delta\ AA111–331}$	This study
230	YNB19	pHL449-1	Tf1-reporter (see above)	pBNB117	*nup124* $^{\Delta\ AA571–1150}$	This study
540	YNB217	pBNB193	*nmt1:Tf1-Gag:YFP*	—		This study
541	YNB281	pBNB193	*nmt1:Tf1-Gag:YFP*	—		This study
557	YNB19	—		pBNB194	*nmt1:CFP*	This study
560	YNB16	—		pBNB198	*nmt1:CFP:HIV-1Vpr*	This study
562	YNB19	—		pBNB198	*nmt1:CFP:HIV-1Vpr*	This study
563	YNB16	—		pBNB194	*nmt1:CFP*	This study
871	YNB16	pBNB 193	*nmt1:Tf1-Gag:YFP*	—		This study
893	YNB19	pHL449--1	Tf1-reporter (see above)	pBNB418	*nmt81(Empty vector)*	This study
895	YNB19	pHL449-1	Tf1 reporter (see above)	pBNB439	*nmt81:Nup124* $^{AA1–1159}$	This study
924	YNB19	pHL449-1	Tf1-reporter (see above)	pBNB469	*nmt81:nup124* $^{\Delta\ AA272–454}$	This study
927	YNB19	pHL449-1	Tf1-reporter (see above)	pBNB470	*nmt81: nup124* $^{\Delta\ AA272–454}$*:: nup153*$^{AA448–634}$	This study
932	YNB16	pHL449-1	Tf1-reporter (see above)	pBNB8	*nmt1:GFP*	This study
933	YNB16	pHL449-1	Tf1-reporter (see above)	pBNB455	*nmt1:nup124*$^{AA264–454}$	This study
934	YNB16	pHL449v1	Tf1-reporter (see above)	pBNB456	*nmt1:nup124*$^{AA264–454}$	This study
935	YNB16	pHL449-1	Tf1-reporter (see above)	pBNB457	*nmt1:nup153*$^{AA447–634}$	This study
936	YNB16	pHL449-1	Tf1-reporter (see above)	pBNB458	*nmt1:nup153*$^{AA447–634}$	This study
909/910	YNB16	pHL449-1	Tf1-reporter (see above)	pBNB443	*nmt81:nup124*$^{AA264–454}$	This study
911/912	YNB16	pHL449-1	Tf1-reporter (see above)	pBNB448	*nmt81:nup153*$^{AA447–634}$	This study
964	YNB19	pBNB223	Empty vector	pBNB492A	*nmt81:Nup153* $^{AA1–1475}$	This study
965	YNB19	pBNB223	Empty vector	pBNB492B	*nmt81:Nup153* $^{AA1–1475}$	This study
966	YNB19	pHL449-1	Tf1-reporter (see above)	pBNB492A	*nmt81:Nup153* $^{AA1–1475}$	This study
967	YNB19	pHL449-1	Tf1-reporter (see above)	pBNB492B	*nmt81:Nup153* $^{AA1–1475}$	This study
962	YNB16	—		pBNB502	*nmt1:SIV Vpr:gfp*	This study
963	YNB19	—		pBNB502	*nmt1:SIV Vpr:gfp*	This study
971	YNB16	—		pBNB508	*nmt1:SIV Gag:gfp*	This study
972	YNB19	—		pBNB508	*nmt1:SIV Gag:gfp*	This study
816	YNB38	—		pBNB507	*nmt41:gfp:SIV Vpx*	This study
810	YNB19	—		pBNB507	*nmt41:gfp:SIV Vpx*	This study

expressed in *S. pombe* along with a Tf1 reporter plasmid, pHL449–1 (Balasundaram *et al.*, 1999) (Figure 2A) to determine the levels of Tf1 cDNA recombination and transposition (Figure 2B) by in vivo complementation of the defect in a null mutant using previously described genetic assays (Balasundaram *et al.*, 1999). In addition, immunofluorescent approaches were applied to visualize these mutant HA-tagged Nup124 proteins inside the cell (Figure 2C). As seen in Figure 2B, deleting either the entire eleven FXFG-repeat domain nup124$^{\Delta AA571–1150}$ or nup124$^{\Delta AA111–333}$ containing all five N-terminal basic amino acid clusters exhibited levels of Tf1 cDNA recombination and transposition comparable to the

Table 2. Plasmid/constructs

Construct	Description	Reference
pBNB12	A triple copy of the HA epitope was generated by PCR using pHL1339-2 as template with HL420 and HL637. The resulting 0.88-kb *Bam*HI-*Bcl*I fragment containing the native promoter region of *nup124* and the 3HA epitope replaced the promoter region between the *Bam*HI-*Bcl*I sites in the *leu2* selectable plasmid pHL1471-2 (Balasundaram *et al*., 1999) so that the ATG codon plus 3HA sequence were introduced in-frame at the N-terminus immediately upstream of the *nup124* coding sequence.	This study
pBNB117	Nucleotides representing AA571–1150 of Nup124p were deleted by generating a PCR product using NB40 + NB41. The 1.4-kb *Avr*II-*Bgl*II fragment containing the AA571–1150 deletion was cloned into BNB12 to replace the region between those same sites.	This study
pBNB119	Nucleotides representing AA1–110 and AA332–980 of Nup124 were generated using HL417 + NBO8 and NBO7 + HL630 sets of primers, respectively. The products of the first round were used for fusion PCR using the primers HL417 + HL630. The resulting *Bcl*I-*Nco*I fragment containing the AA111–331 deletion was cloned into BNB12 to replace the region between those same sites.	This study
pBNB193	The yellow fluorescent protein (YFP) was generated with primers NB383 and NB384. The resulting 0.7-kb fragment was cloned into the *Ngo*MIV site at the N-terminus of Gag in pHL1258 (Balasundaram *et al*., 1999). The ability of BNB193 to generate wild type levels of Tf1 activity in Tf1 transposition assays confirmed the functional competency of the Tf1 transposon (see *Materials and Methods*).	This study
pBNB194	Cyanin fluorescent protein (CFP) was amplified using primers NB381 and NB382 and cloned into the *Not*I-*Bgl*II sites of pREP1 downstream from the *nmt1* promoter.	This study
pBNB327	pcDNA3	Invitrogen
pBNB349	Tf1-Gag was amplified as FLAG-tagged-Gag fusion using NB802 and NB803 and cloned into the mammalian expression vector pCDNA3 as an *Eco*RI-*Xho*I fragment.	This study
pBNB 400	A 2.2-kb fragment representing the ORF SPAC1B1.03c, encoding the importin β, Kap95 (Chen *et al*., 2004) was generated using NB985 as forward and NB 984 as reverse primers and cloned into pET41b as a *Kpn*I-*Not*I fragment so as make a GST-6His-S. Tag-Kap95 fusion product.	This study
pBNB401	The entire *nup124* (*Bam*HI-*Eco*RV) coding region was PCR-amplified using NB789/NB757 and cloned downstream of the T7 promoter in the mammalian expression vector pCDNA3.	This study
pBNB405	Full-length huNup153 in pcDNA1	Gift from Michael J. Matunis
pBNB422	HIV-1 *Vpr* coding region from the infectious dual tropic clone of HIV-$1^{89.6}$ was PCR-amplified as a *Hin*dIII-*Xho*I fragment using NB737/NB738 and cloned downstream of the T7 promoter in the mammalian expression vector pCDNA3 to generate a C-terminal FLAG epitope-tagged HIV-1 *Vpr*.	This study
pBNB429	The Gag coding region from the infectious simian immunodeficiency virus Pbj1.9 was PCR-amplified as a *Eco*RI-*Xho*I fragment and cloned downstream of the T7 promoter in the mammalian expression vector pCDNA3 to generate a N-terminal FLAG epitope-tagged SIV-Gag.	This study
pBNB430	The *Vpr* coding region from the infectious simian immunodeficiency virus Pbj1.9 was PCR-amplified as a *Hin*dIII-*Xho*I fragment and cloned downstream of the T7 promoter in the mammalian expression vector pCDNA3 to generate a N-terminal FLAG epitope-tagged SIV-*Vpr*.	This study
pBNB439	*nmt81:Nup124$^{AA1–1159}$* The full-length gene encoding Nup124$^{AA1–1159}$ was cloned from pBNB401 as *Bam*HI-*Eco*RV fragment into the *Bam*HI-*Sma*I sites of pCDL28 so that *Nup124* was transcribed from an *nmt81* promoter.	This study
pBNB469	*nmt81:nup124Δ $^{AA272–454}$* Nucleotides representing AA112–271 and AA455–980 of Nup124 were generated with BNB401 as template using NB1134 + NB1135 and NB1136 + NB1137 sets of primers respectively. The products of the first round were used for fusion PCR using the primers NB1134 + NB1137. The resulting 2.0-kb *Avr*II-*Nco*I fragment containing the AA272–454 deletion was cloned into BNB439 to replace the region between those same sites.	This study
pBNB470	*nmt81:nup124Δ $^{AA272–454}$::nup153AA448–634* Nucleotides representing AA112–271 and AA455–980 of Nup124 were generated with BNB401 as template using NB1134 + NB1141 and NB1142 + NB1137 sets of primers, respectively. A fragment representing AA448–634 of Nup153 was generated using NB1121 and NB1122 with BNB405 as template. The NB1121 and NB1122 had 100 bases of Nup124 flanking the deletion on the 5′ and 3′ ends, respectively. The three products of the first round were used for fusion PCR using the primers NB1134 + NB1137. The resulting 2.6-kb *Avr*II-*Nco*I fragment containing the *nup124$^{AAA272–454}$* deletion with *Nup153$^{AA448–634}$* fragment in-frame insertion was cloned into BNB439 to replace the region between those same sites.	This study
pBNB443/ 455/456	*(nmt81-) and (nmt1)-Nup124$^{AA264–454}$* A 0.54-kb product was generated by PCR using pBNB401 as template with NB1029 and 1030 as forward and reverse primers, respectively. The PCR product was cloned into a pNMT81-TOPO vector (Invitrogen) to yield pBNB443 (*pNMT81-TOPO-Nup124$^{AA264–454}$). A Bgl*II-*Sal*I fragment from BNB443 containing the Nup124 sequence with an in-frame V5 epitope tag was cloned into *Bam*HI-*Xho*I sites replacing *gfp* downstream from the *nmt1* promoter in pCDL280 to yield BNB455 and 456 (*pnmt1-Nup124$^{AA264–454}$*).	This study
pBNB448/ 457/458	*(nmt81-) and (nmt1)-Nup153$^{AA447–634}$* A 0.55-kb product was generated by PCR using pBNB405 as template with NB1027 and 1028 as forward and reverse primers, respectively. The PCR product was cloned into a pNMT81-TOPO vector (Invitrogen) to yield BNB448 (pNMT81-TOPO-Nup153$^{AA447–634}$). A *Bgl*II-*Sal*I fragment from BNB448 containing the Nup153 sequence with an in-frame V5 epitope tag was cloned into *Bam*HI-*Xho*I sites downstream from the *nmt1* promoter in pCDL280 to yield BNB457 and 458 (*pnmt1-Nup153$^{AA447–634}$*).	This study
pBNB492	*nmt81:Nup153$^{AA1–1475}$* The full-length gene encoding Nup153$^{AA1–1475}$ was cloned from pBNB405 into the *Not*I-*Xho*I sites of pBNB448 to yield *nmt41:Nup153:V5:6xHis*	This study
pBNB502	SIV-*Vpr* was generated by PCR with primers NB1079 and NB1080 using pBNB429 as template and cloned into the pREP1 plasmid downstream of the *nmt1* promoter between *Sal*I-*Not*I sites to yield *nmt1:SIV-Vpr:GFP*	This study
pBNB507	SIV-Vpx was cloned from pBNB496 (SIV-Vpx in pcDNA3) into the *Nde*I-*Bam*HI sites of pREP-41-NT-GFP to yield *nmt41:GFP:SIV-Vpx*	This study
pBNB508	SIV-Gag was amplified from pBNB430 as a 1.5-kb fragment using primers NB1081 and NB 1082 and cloned into the pREP1 plasmid downstream of the *nmt1* promoter between *Sal*I-*Not*I sites to yield *nmt1:SIV-Gag: GFP*.	This study
pCDL28/ pBNB418	pREP3X-*nmt81*	Gift from Mohan Balasubramanian
pCDL280/ pBNB8	pREP1 with *gfp* (S65T)	Gift from Mohan Balasubramanian

Table 3. Oligonucleotides

Number[a]	Sequence (5′-3′)
HL420^{F}	CATCGTAAGGATCCATTTGTCTTCCACCAATTTTTGAATT
HL637^{R}	ATGTGTAGGTTAAGTGATCAAGGCGTAATCAGGCACATCATATGGGTAGGCGTAATCAGGCACATCATATGGG-TAGGCGTATCAGGCACATCATATGGGTACATCAATACAAACTCATCGACAACTAC
HL417 ^{F}	GTCGATGAGTTTGTATTGATCACTCCTGTTTCAAAAAATAC
NBO8 ^{R}	GACCTTTCTCGAGAGTCTGTTCTTATGTATTCGTATGAAC
NBO7 ^{F}	GTTCATACGAATACATTTGAACAGACTCTCGAGAAAGGTC
HL630 ^{R}	CAAAAGTTACCTAGATCTTAACCAAACATAGAAGCAGGC
NB40 ^{F}	CATTTGAACCTAGGAAGCC
NB41 ^{R}	ACCTAGATCTTAACGTTTTCTTCGACTTCGACCAGAAGTTGAAGCAGGAG
NB383 ^{F}	GATCATGCAGCCGGCAGTAAAGGAGAAGAACTT
NB384 ^{R}	TTACT CGCCGGCTTTGTATAGTTCATCATCCATGCC
NB381 ^{F}	GATCCGCGGCCGCAGATGAGTAAAGGAGAAGAACTT
NB382 ^{R}	TTACTCGAGATCTTTGTATAGTTCATCATCCATGCC
NB387 ^{F}	TACAAAGATCTCATGGAACAAGCCCCAGAA
NB388 ^{R}	GTATAGCCCGGGC TAGGATTTACTGGCTCC
NB789^{F}	TCCATCGGATCCGCCGCCACCATGGACTACAAGGATGACGATGACAAAATGC-CTCCTGTTTCAAAAAATACC
NB757 ^{R}	TTTAGCGATATCTTAACGTTTTCTTCGACTTCGGGG
NB753 ^{F}	CGTCAGACTCTCGAGAAAGGTCATATT
NB754 ^{R}	AATATGACCTTTCTCGAGAGTCTGACG
NB737 ^{F}	ATCTCGAAGCTTGCCGCCACCATGACAGAAAGACCTCCAGAAGAT
NB738 ^{R}	GTCTAGCTCGAGCTAGGATTTACTGGCTCCATTTCTTGTTC
NB802 ^{F}	ACTGCGAATTCGCCGCCACCATGAAAAACTCATCACAGAAAAGGATTCGAATGGATGGAAATGG
NB803 ^{R}	AGCTGCTCGAGAATAACGTCTTTTCTTGTATTTTG
NB984^{F}	GACTACGGTACCATGAACGCAGGTGAG
NB985^{R}	CATCTGCGGCCGCGGAGCTCTAGCTTGACGCTT
NB1121 ^{F}	AAATGATTCCCATAAGAGTCTTACCGACATTAGAGATAAGGAGAATGGCGAAACAGAGGTCTCAGCAAAAAA-TCACGTTCCTCACCGCTCCTCTCGGCGAAAGATGAGACGAGAAAGAAC
NB1122^{R}	CTTCAGTCTTCGGTTCAAACTTAAATTCGGGTACTGAAAAGTGAGAAGATTTGATTTCCTTAGAAGGTGAT-GGCGGTTCAACTTCGGACTTTCTAGTTGTTGGGCTTGTTGCGGTGGGCT
NB1134 ^{F}	CATTTGAACCTAGGAAGCC
NB1135^{R}	TTCGGACTTTCTAGTTGTTCGCCGAGAGGAGCGGTG
NB1136 ^{F}	CACCGCTCCTCTCGGCGAACAACTAGAAAGTCCGAA
NB1137^{R}	CTTAGCCATGGGAGTGTTCTC
NB1141^{R}	TCGCCGAGAGGAGCGGTGAGGAACGTGATTTTTTGC
NB1142^{F}	ACAACTAGAAAGTCCGAAGTTGAACCGCCATCACCT
NB1079^{F}	TACTGGTCGACTATGACAGAAAGACCTCC
NB1080^{R}	AGTCCGCGGCCGCGGTAACACGCCTCT
NB1081^{F}	TACTGGTCGACTATGGGCGCGAGAAAC
NB1082^{R}	AGTCCGCGGCCGCGGTTGGTCTTCTCCAAAG
NB1027^{F}	GCTGCATATGCAGATCTCCGCGGCCGCGGTATGGGCAAGATGAGACGAGA
NB1028^{R}	GCTCGAGCGCCCGGGTGGGCTTGTTGCGGTG
NB1029^{F}	GCTCGGTACCGGCCGGATCCGCCAGATCTCGGAATGGTTCCTCACCGCTCCT
NB1030^{R}	GCGGCC/GCGGAT/ATCAGATTCTTTTGTCGGCGA

[a]F, forward primer; R, reverse primer.

null mutant suggesting that both domains were independently required for Tf1 activity. Next, we asked whether loss of Tf1 transposition activity observed in these mutants was caused by altering their ability to localize to the NPC. Strains with plasmids expressing 3HA:Nup124p and mutants 3HA:nup124p$^{\Delta AA571-1150}$ or 3HA:nup124p$^{\Delta AA111-333}$ (Figure 2C) were examined for localization by indirect immunofluorescence using a monoclonal antibody (mAb) to the HA epitope. Both mutant strains exhibited similar pattern of perinuclear staining when compared with the wild-type. It is therefore possible that in the absence of the either domain, Tf1 VLPs are recruited to the NPC but unable to execute the subsequent steps of the translocation mechanism.

Activity of HIV-1 Vpr in S. pombe *Is Nup124p-dependent*

HIV-1 Vpr is known to localize to the nuclear envelope in mammalian cells (Mahalingam *et al.*, 1995a; Vodicka *et al.*, 1998), *S. cerevisiae* and *S. pombe* (Zhao and Elder, 2000; Chang *et al.*, 2004). Because Nup124p localizes to the nuclear envelope in *S. pombe* and is required for the nuclear import of Tf1-Gag (Balasundaram *et al.*, 1999), we asked if Nup124p was one of the targets of Vpr at the NPC. We therefore constructed a plasmid expressing HIV-1 CFP:Vpr (BNB198) whose expression in *WT* and Δ*nup124* strains of *S. pombe* was under the control of the thiamine-repressible promoter, *nmt1*. The HIV-1 Vpr was amplified from the pathogenic dual tropic clone 89.6 of HIV-1 (Mahalingam *et al.*, 1997a). In the presence of thiamine, cells showed no inhibition in growth (Figure 3A, left panel). When HIV-1 Vpr expression was induced, wild-type cells were growth-arrested and died subsequently, whereas the Δ*nup124* cells survive (Figure 3A, right panel). To test whether a certain population of cells lacking Nup124p had a distinct growth advantage when Vpr was expressed, we grew up cultures in thiamine and spread 1×10^3 and 1×10^6 cells on plates containing thiamine. After 2 d, these plates were printed to ones without thiamine to

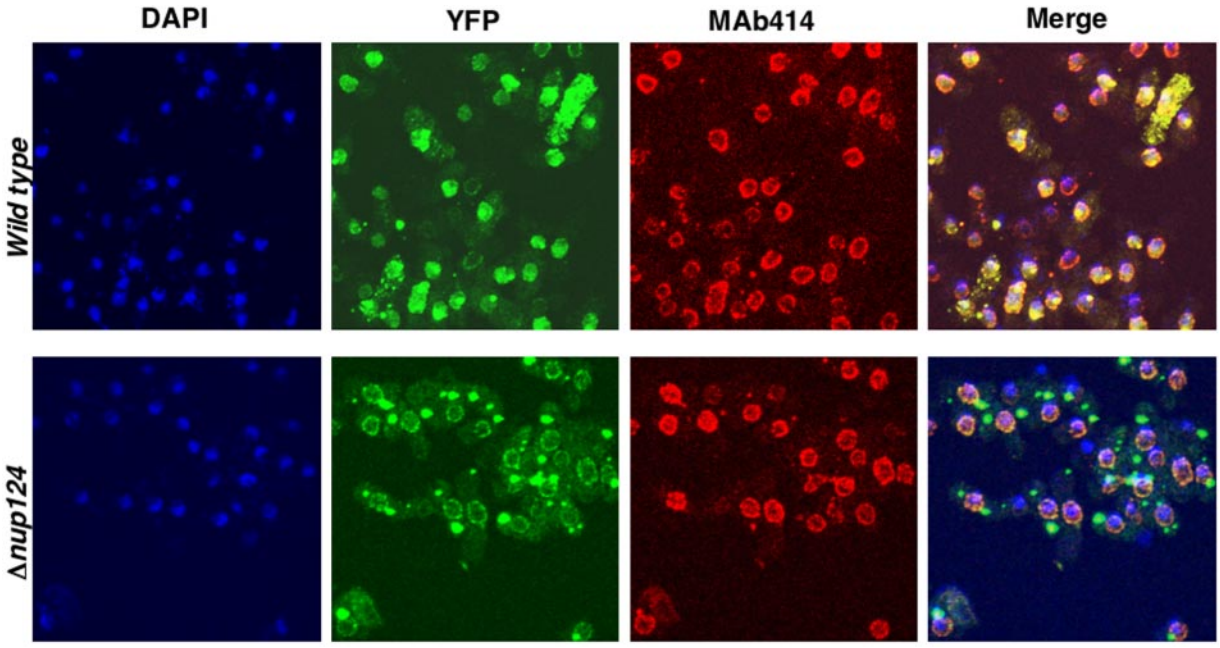

Figure 1. Nup124 is required for translocation of Tf1-Gag from the NPC into the nucleus. An inducible YFP-tagged Tf1-Gag construct was expressed in multicopy plasmids in *WT* (YNB540, top) and Δ*nup124* (YNB541, bottom) host strains. The nuclear envelope and Tf1-Gag:YFP were visualized by indirect immunofluorescence using MAb414 and GFP antibodies that react with FXFG-containing nucleoporins and YFP, respectively (GFP polyclonal antibody recognizes YFP). Nuclear DNA was stained with DAPI. Strains were grown overnight to saturation (stationary phase) in the absence of thiamine to induce *nmt1*-Tf1 expression. Images were acquired through a 100× objective lens using a Zeiss LSM 510 confocal microscope. Cells were imaged in *Z*-series and deconvolved. MAb414 (red) staining was used to fix the upper and lower limits of the *Z*-series. Figure depicts a single plane *Z*-section passing through the center of the *Z*-series.

induce Vpr expression. When plates incubated for 4–6 d at 32°C were examined for growth, we observed that wild-type cells did not form colonies, whereas the null mutant formed a titratable number of colonies (Figure 3B). Our results demonstrate that Nup124p may be one of the targets of Vpr at the NPC because absence of the nucleoporin supports survival of the host strain. Another possibility is that Nup124p allows Vpr access to critical nuclear components required for cell growth and viability. In the absence of Nup124p, Vpr may be mislocalized, so its interactions or effects on nuclear components may be altered or lost.

HIV-1 Vpr Is Mislocalized in the Absence of Nup124p

Because absence of *Nup124* abrogated Vpr-mediated growth inhibition and cell death, we asked whether the localization of HIV-1 Vpr was altered in *WT* and Δ*nup124* strains. *WT* and Δ*nup124* strains expressing the CFP:Vpr fusion protein were examined by confocal microscopy. Vpr was localized at the nuclear envelope (NE) with the FXFG containing nucleoporins (stained with MAb414) both in *WT* as well as the Δ*nup124* strains (Figure 4). However, significant differences in the localization patterns were observed. Accumulation of CFP:Vpr signal appeared usually in the form of a "bleb" or blister in the wild strain exclusively. *Z*-stack and projection images showed that this bleb characteristically appeared on the inner side of the nuclear envelope and was embedded within the MAb414 staining (Figure 4, top panel, and online Supplementary Video YNB560Z-Stack.MOV). In contrast, no blebs were visible within the nucleus of the mutant. Instead, the NE formed projections into the cytoplasm (Figure 4, bottom panel, and online Supplemmentary Video YNB562Z-Stack.MOV). Indeed, *Z*-series images confirmed those observations (see Supporting Online Material). Like Tf1-Gag, the HIV-1 Vpr is recruited to the NE, but may be mislocalized in the absence of Nup124p and thus, may not have access to the inner face of the nucleus. Such a result may be consistent with our observation in Figure 3 that absence of Nup124p abrogates loss of cell viability and growth.

Nup124p Interacts with Tf1-Gag and HIV-1 Vpr In Vitro

Our genetic and localization data imply a relationship between Nup124p and Tf1-Gag or HIV-1 Vpr. To determine whether Vpr and Tf1-Gag were capable of physically interacting with Nup124p, we performed coimmunoprecipitation experiments using individually translated products from rabbit reticulocyte lysates in vitro. DNAs encoding Nup124p, HIV-1 Vpr, SIV-Vpr, Tf1-Gag, and SIV-Gag (Table 2) were individually transcribed and translated in the presence of [^{35}S]methionine. Figure 5A shows the yield and size of the products when 1.0-μl aliquots of the synthesized protein were separated on an 8–16% SDS-PAGE gel. We noted that in each case the synthesized products corresponded to the expected molecular size as indicated. However, we do see some additional species that were possibly incomplete translational products. Approximately equimolar amounts of each translation product shown in Figure 5A were mixed in various combinations and immunoprecipitated with antibodies as indicated in Figure 5B. Immunoprecipitates were analyzed on an 8–16% gradient SDS-PAGE gel and subjected to fluorography. Figure 5B shows that a mixture of Nup124p and Tf1-Gag:FLAG was immunoprecipitated by either anti-FLAG (lane 1) or the MAb414 (lane 2). Similarly, a mixture of Nup124p and HIV-1 Vpr were immunoprecipitated by either the MAb414 (lane 3) or anti-Vpr (lane 4). To exclude the possibility of nonspecific inter-

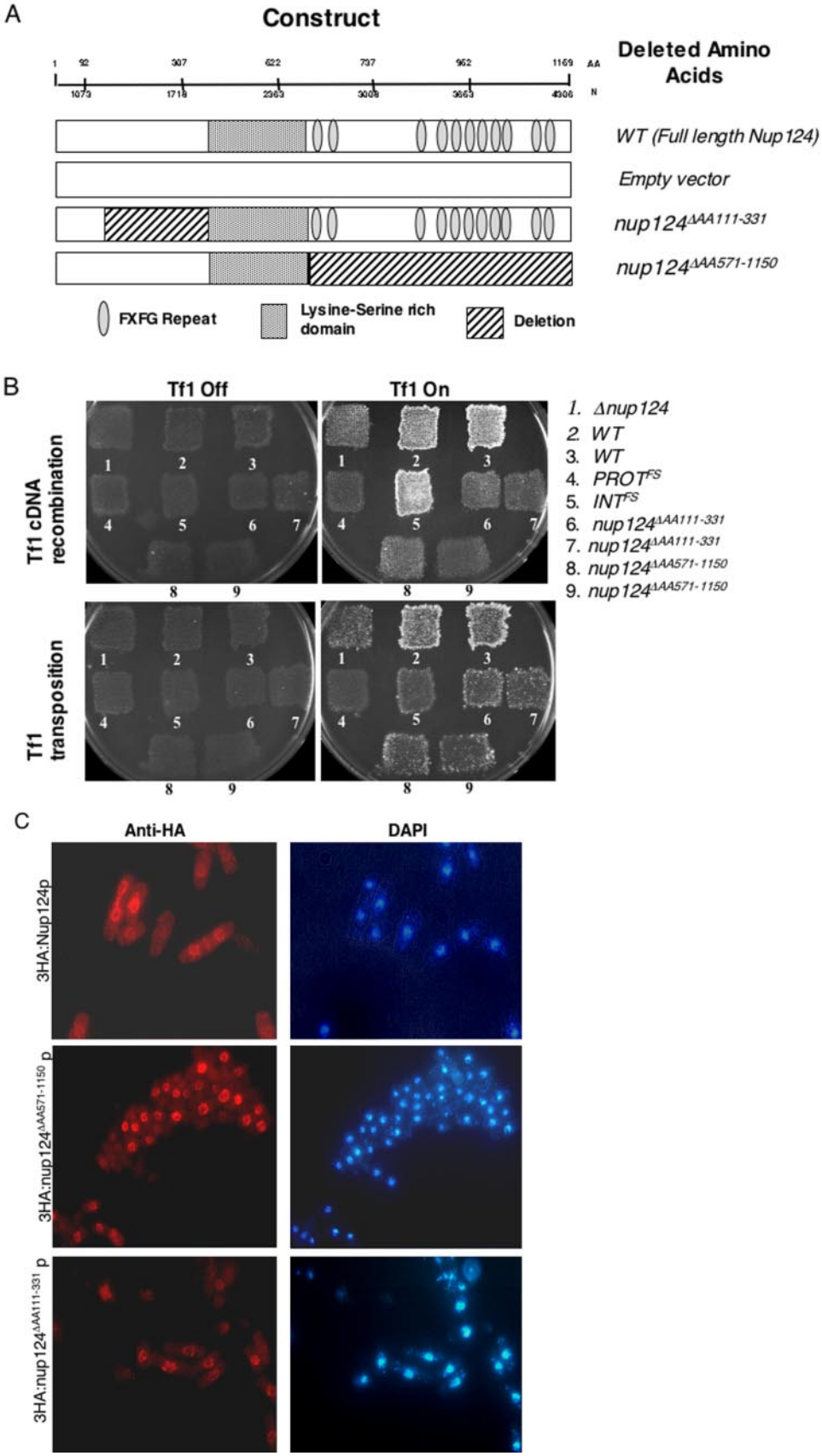

Figure 2. Functional domains of Nup124. (A) Multicopy plasmids containing full-length and mutant versions of *Nup124* whose transcription was under the control of its native promoter were transformed into a Δ*nup124* mutant strain YNB19 along with the Tf1 reporter plasmid pHL 449-1. (B) Previously described genetic assays (Balasundaram *et al.,* 1999) measured the ability of strains containing epigenetically expressed *WT* (YNB58) and *mutant* versions of nup124 (nup124$^{\Delta AA111–331}$, YNB244 and nup124$^{\Delta AA571–1111}$, YNB230) to reinstate levels of Tf1 cDNA recombination and transposition in a Δ*nup124* mutant (YNB8) to that of endogenous or epigenetically expressed *WT* levels. PROT^{fs} (YNB10) contains a Tf1-reporter plasmid with a protease frameshift that blocks the expression of (Tf1) protease, reverse transcriptase, and integrase and serves as a negative control for the assay. Because protease is involved in the processing of the Tf1 genome, all the activities of the retrotransposon are blocked as a result of the frameshift mutation. IN^{fs} (YNB11) contains a Tf1-reporter plasmid with a frameshift in the integrase so Tf1 transposition is decreased but not Tf1 cDNA recombination. (C) Cellular localization of Nup124p. The Δ*nup124* mutant expressing the full-length (3HA:Nup124, YNB58) or lacking the N-terminal domain (3HA: nup124$^{\Delta AA111–331}$, YNB244) or FXFG-repeat domain (3HA: nup124$^{\Delta AA571–1150}$, YNB230) were prepared for immunofluorescence microscopy as given in *Materials and Methods.* The red signal in the panels on the left are specific for the HA-tagged proteins by virtue of their immunoreaction with the anti-HA MAb, 12CA5.

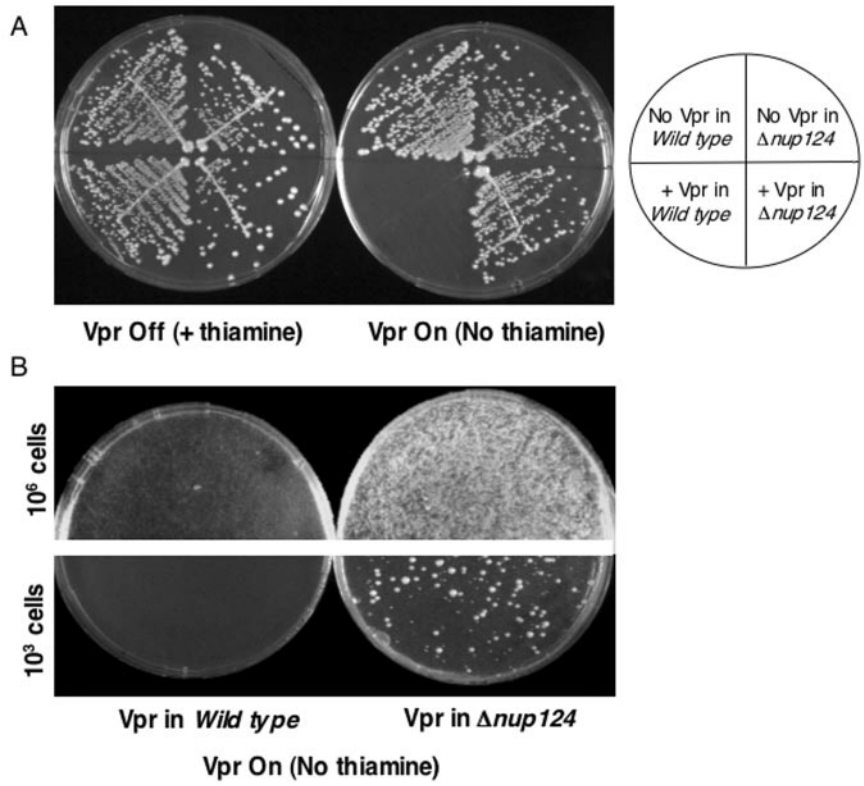

Figure 3. HIV-1 Vpr activity in *S. pombe* is Nup124p-dependent. (A) Absence of Nup124p enables cells to be more resistant to Vpr-induced cell killing activity. *WT* (YNB560) and Δ*nup124* (YNB562) cells expressing HIV-1 Vpr were grown overnight in medium containing 15 mM thiamine (Vpr expression-off) and washed twice in medium lacking thiamine. "Washed cells" were used as the source to be streaked for single colonies on plates with (Vpr expression-off) or without (Vpr expression-On) 15 μM thiamine. The top sectors of plates contained *WT* and Δ*nup124* strains with control plasmids lacking Vpr (YNB563 and YNB557, respectively). (B) To ascertain whether Δ*nup124* cells had a growth advantage over *WT* expressing HIV-1 Vpr, the washed cells described above were spread on plates at the indicated cell densities without thiamine to induce for expression of Vpr. Plates were photographed after 5 d incubation at 32°C.

actions, we used Gag and Vpr or Vpx from the SIV belonging to the same family of lentiviral proteins. To ascertain whether the nuclear import of SIV-Gag, SIV-Vpr, or SIV-Vpx was not dependent on Nup124p, they were expressed from a thiamine-repressible reporter, *nmt1* as C-terminal fusions (Table 2) with GFP and transformed into *WT* and Δ*nup124* strains (Table 1). Microscopic examination revealed that localization of SIV-Gag:GFP was cytoplasmic whereas SIV-Vpr:GFP and SIV-Vpx:GFP were nuclear irrespective of the absence or presence of Nup124p (unpublished data). We reasoned therefore that those viral proteins would be appropriate negative controls. Plasmids expressing SIV-Gag:FLAG, SIV-Vpr:FLAG, and SIV-Vpx:FLAG were individually transcribed and translated in the presence of [^{35}S]methionine (Figure 5A). Figure 5B, lanes 5 and 6, show Nup124p does not coimmunoprecipitate with SIV-Gag:FLAG. However, when a mixture of Nup124p and SIV-Vpr:FLAG is treated with MAb414, a 14-kDa band correspond-

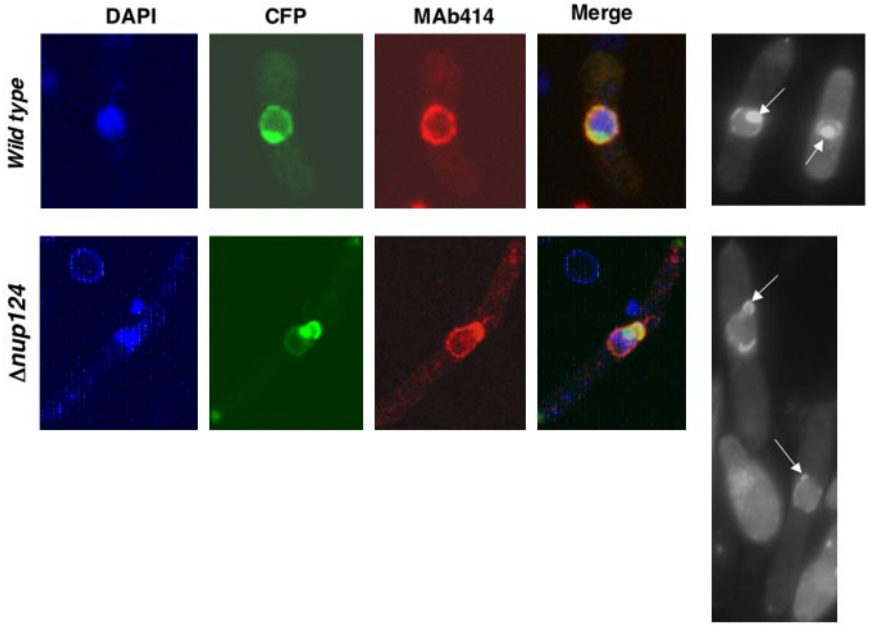

Figure 4. Localization of HIV-1 Vpr in the presence and absence of Nup124p. *WT* (YNB560, top) and Δ*nup124* (YNB562, bottom) strains expressing HIV-1 CFP:Vpr under the control of an *nmt1* promoter were grown in the absence of thiamine and prepared for immunofluorescence as described in *Materials and Methods.* The nuclear envelope and HIV-1 CFP:Vpr were visualized by indirect immunofluorescence with MAb414 antibody that specifically reacts with FXFG-containing nucleoporins and *α*nti-GFP antibody, respectively. Nuclear DNA was stained with DAPI. Arrows indicate nuclear blebs or cytoplasmic projections. Experimental and image-capture conditions were identical to those detailed in Figure 1. Figure shown depicts a single plane *Z*-section passing through the center of the series.

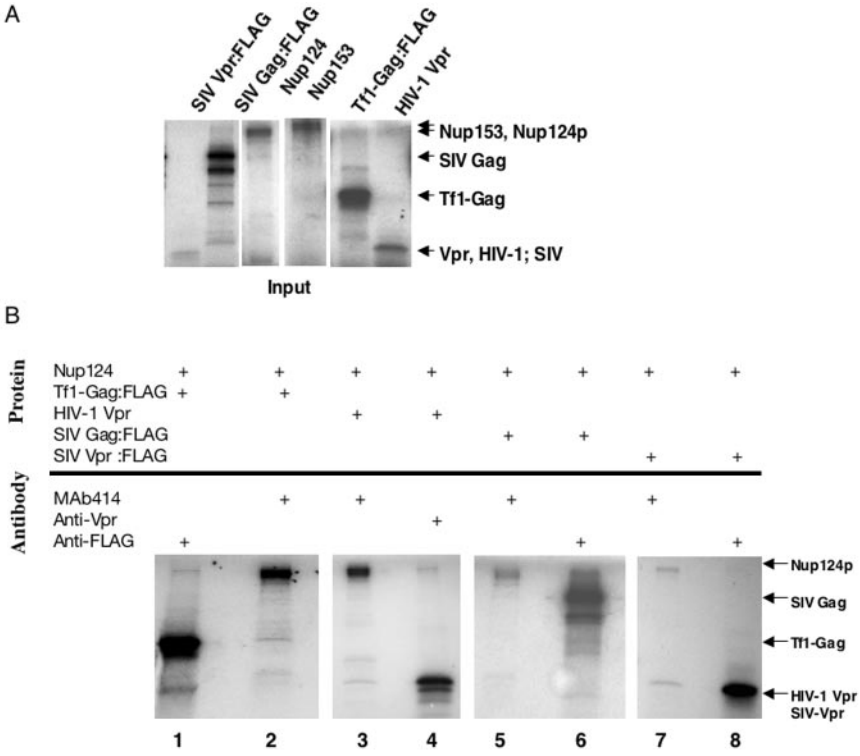

Figure 5. Coimmunoprecipitation of Nup124p with Tf1-Gag and HIV-1 Vpr. (A) Proteins were transcribed and translated in rabbit reticulocyte lysates in the presence of [^{35}S]methionine. (B) Translation products were mixed in various combinations as indicated. Coimmunoprecipitation assays were performed as described in *Materials and Methods* with the appropriate antibodies as indicated. The immunoprecipitates were resolved on 8–16% SDS-PAGE and detected by fluorography. The relative mobilities of full-length/+tag proteins are indicated by arrows.

ing to SIV-Vpr is observed with MAb414 and a very faint band with anti-FLAG (lanes 7 and 8, respectively), suggesting there might be a weak interaction between Nup124p and SIV-Vpr. No interaction was observed between Nup124p and SIV-Vpx (unpublished data).

Search for a Mammalian Homolog of Nup124p

Because Nup124p interacted with Tf1-Gag and HIV-1 Vpr in vitro and because of the latter's possible role in AIDS pathogenesis (Sherman *et al.*, 2002b; Tungaturthi *et al.*, 2003), we asked if a human homolog of the nucleoporin might do the same. A BLASTp search revealed three FXFG-containing proteins that share a low similarity with the *S. pombe* Nup124p (Figure 6A). They were the *S. cerevisiae* (Sc) nucleoporins ScNup1p, ScNsp1p, and the vertebrate (human) nucleoporin huNup153p. Proteomic analysis of mammalian NPC proteins and comparison with those from *S. cerevisiae* previously identified Nup1p as a homolog of Nup153 (Stoffler *et al.*, 1999; Cronshaw *et al.*, 2002). Interestingly, all three proteins contain very large numbers of FG-repeats scattered over the entire length of the protein but differ in the position of FXFG-repeat domains. Nup153 resembles Nup124p closely in that the FXFG-repeat domain is at the C-terminus, whereas for Nup1p it is in the middle and Nsp1p, at the N-terminus (Figure 6A). Comparison of Nup124p and Nup153 sequences revealed a significant number of short conserved stretches of residues at the N and C-termini (Figure 6, B and C). We asked therefore, if Nup153 was able to interact with both Tf1-Gag and HIV-1 Vpr in vitro just as Nup124p did. Equal amounts of in vitro translated Nup153, HIV-1 Vpr, and Tf1-Gag proteins were subjected to a coimmunoprecipitation assay using MAb414, anti-Vpr, or anti-FLAG antibodies as described for Figure 5. Figure 7 shows that a mixture of Nup153 and Tf1-Gag:FLAG was immunoprecipitated by either anti-FLAG (lane 1) or the MAb414 (lane 2). Similarly, a mixture of Nup153 and HIV-1 Vpr were immunoprecipitated by either the MAb414, (lane 3) or anti-Vpr (lane 4). As negative controls, [^{35}S]methionine SIV-Gag:FLAG and SIV-Vpr:FLAG, or SIV-Vpx:FLAG were individually incubated with Nup153. As observed for Nup124p, no immunoprecipitate is recovered with either MAb414 or anti-FLAG when treated with SIV-Gag:FLAG (lanes 5 and 6, respectively), whereas faint bands were observed with SIV-Vpr:FLAG (lanes 7 and 8). No interaction was observed between Nup153 and SIV-Vpx (unpublished data). Our data therefore show that Nup153p interacts with HIV1-Vpr and Tf1-Gag in a manner identical to that of Nup124p. Lane 9 depicts the positive control interaction between SIV-Gag and SIV-Vpr. Because both nucleoporins Nup124p and Nup153 localize to the NPC we wanted to eliminate the possibility that any *non*-nucleoporin that also localizes to the NPC would interact with Tf1-Gag or HIV-1 Vpr. The *S. pombe* Kap95 is an importin β that concentrates at the nuclear rim (Chen *et al.*, 2004). We cloned Kap95 into an expression vector pET41b so as to generate (BNB400, Table 2) an N-terminally tagged GST-6His-*S*.Tag-Kap95 fusion product. [^{35}S]methionine-GST-6His-*S*.Tag-Kap95 treated with [^{35}S]HIV-1 Vpr or [^{35}S]Tf1-Gag was subjected to a coimmunoprecipitation assay using anti-FLAG (Figure 7B, lane 1) or anti-Vpr (Figure 7B, lane 2) exactly as described for Figure 5. No immunoprecipitate was recovered with anti-FLAG, suggesting that Kap95 did not interact with Tf1-Gag. Interestingly, Kap95 did interact with HIV1-Vpr (lane 2). The above reactions were also conducted with anti-His, confirming the above observation (unpublished data).

A Unique Domain of Nup153 Is Absolutely Essential for Tf1 Activity in a nup124 Mutant

Because we had previously demonstrated an N-terminal Nup124p-Tf1-Gag interaction in a yeast two-hybrid analysis (Balasundaram *et al.*, 1999) and in this report both Nup124p and Nup153 independently interacted with Tf1-Gag in vitro,

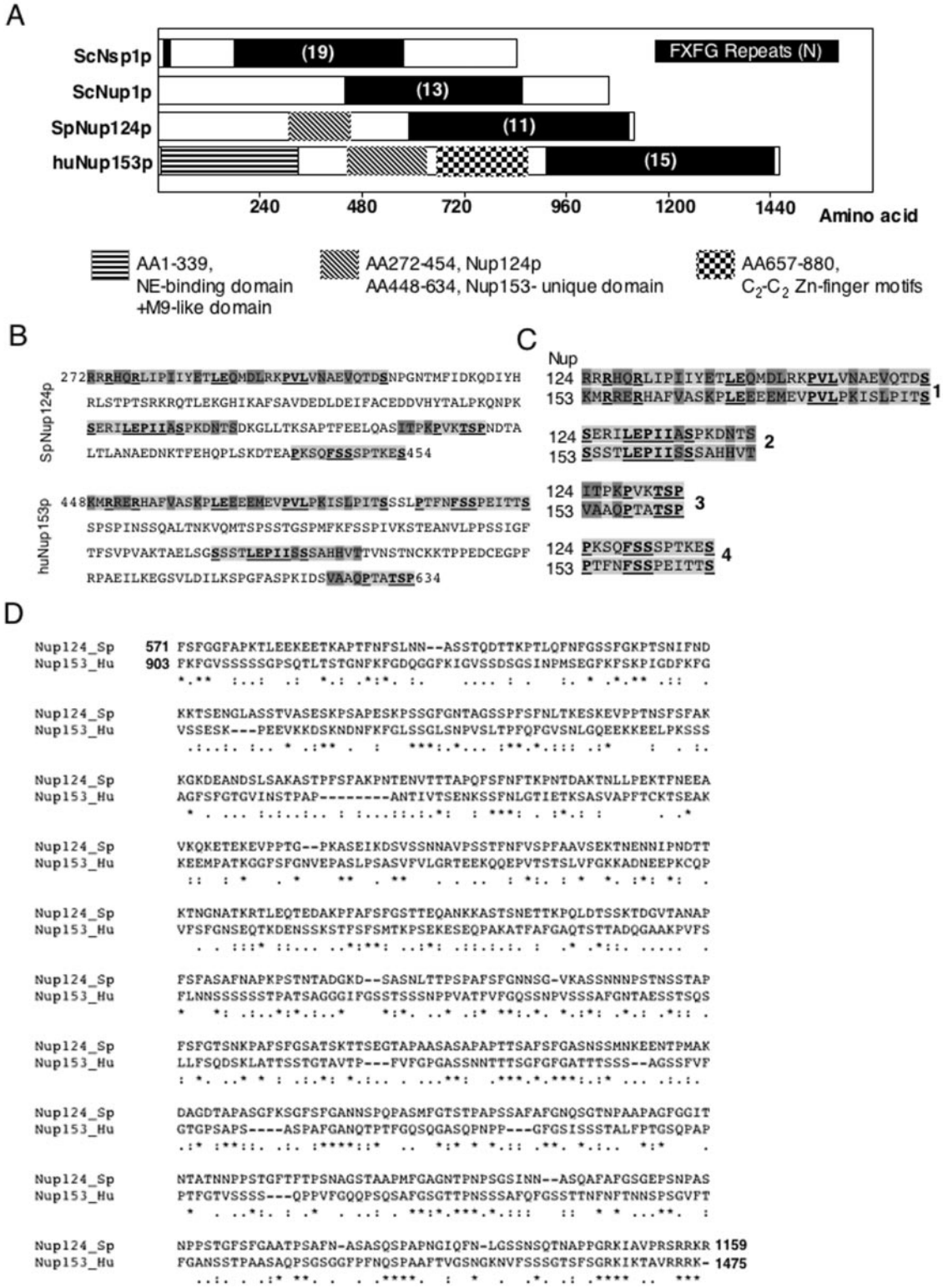

Figure 6. Schematic representation of ScNsp1p, ScNup1p (from *S. cerevisiae*), SpNup124p (from *S. pombe*), huNup153 (from human), and sequence of the conserved domain(s) in SpNup124p and HuNup153. (A) Schematic diagram showing the relative positions of the FXFG-repeat and other unique and conserved domains of Nup153 and Nup124p (see text for details). (N) denotes the number of the FXFG-repeats within the domain. (B–C) The Nup124p amino acid sequence from the N-terminal (AA 272–454) region that is similar to the N-terminal region (AA 448–634) of huNup153. Shaded stretches of amino acids are numbered boxes 1–4 and were aligned using the ClustalW program. Identical residues are underlined and in boldface type, and similar residues are boxed in dark gray. (D) The alignment of the C-terminal (FXFG) domain of the indicated proteins. Asterisk (*), identical residues; colon (:), highly conserved residues; period (.), less conserved residues. Amino acid ranges are indicated at the start and finish of each peptide sequence.

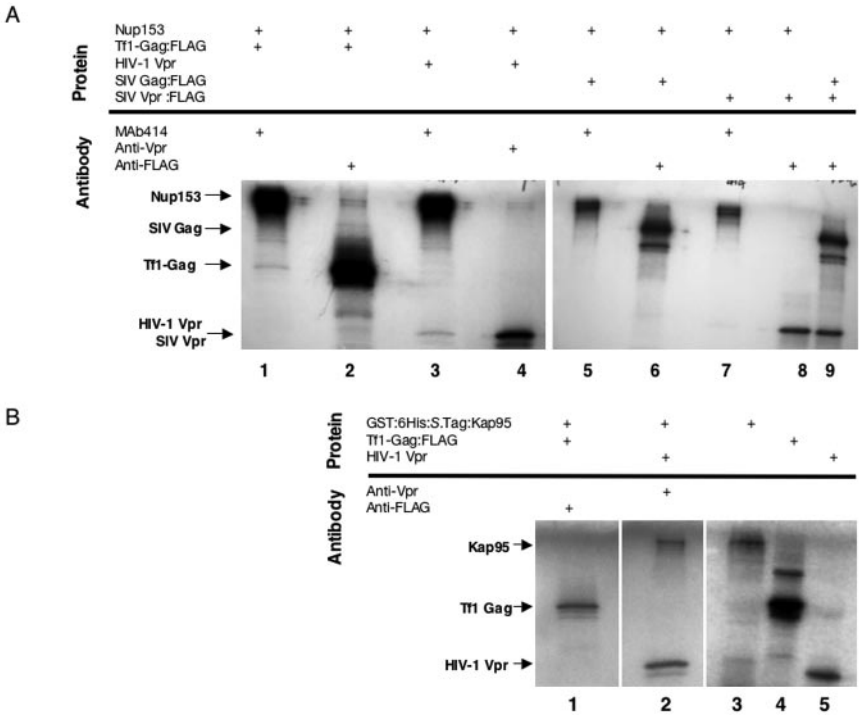

Figure 7. Coimmunoprecipitation of Nup153 with Tf1-Gag and HIV-1 Vpr. (A) Indicated proteins were transcribed and translated in rabbit reticulocyte lysates in the presence of [^{35}S]methionine as depicted in Figure 5A. Translated products as shown were mixed in equimolar amounts in the indicated combinations. Coimmunoprecipitation and all other experimental conditions were identical to those for Figure 5. (B) Coimmunoprecipitation of Kap95 with Tf1-Gag and HIV-1 Vpr. ^{35}S-labeled proteins (lanes 3–5) were transcribed and translated in rabbit reticulocyte lysates as depicted in Figure 5A. Translated products were mixed in equimolar amounts in the indicated combinations. Coimmunoprecipitation and all other experimental conditions were identical to those for Figure 5.

we asked if the Tf1-Gag-binding activity was generated by a homologous domain within these two nucleoporins. The human Nup153 is a large nucleoporin comprising three domains (McMorrow *et al.*, 1994). The N-terminal domain (AA1–339) that has NE-binding activity (Enarson *et al.*, 1998) and an M 9-like domain (Nakielny *et al.*, 1999). The middle domain contains four C_2-C_2 type zinc-finger motifs (AA657–880) comprising the Ran-GDP–binding domain. Nup124p on the other hand, lacks at least the M 9-like domain and C_2-C_2 zinc-finger motifs domains (Figure 6A). Nup153 has a C-terminal domain (572 residues) comprising all 15 FXFG-repeats (AA903–1475). Similarly, all 11 FXFG repeats of Nup124p are in the 588-residue C-terminal domain (AA571–1159) (Figure 6D). The number of identical residues within the FXFG region was significantly high, although no significant alignment of FG-/FXFG repeats was evident (Figure 6D). Interestingly, Nup124p shares an unrecognizable domain that lies between the defined M9-like (AA235–300) and Zn-finger (AA657–880) regions of Nup153 (Figure 6, B and C). Using consensus amino acid residues from each of boxes 1–4 (Figure 6C) to scan protein sequences shown in Figure 6A, we asked whether similar subdomain structures were present. We noted that boxes 1–4 were absent in Nsp1p, Nup1p, and CAN/Nup214 and that box 2 was found only in Nup124p and the human Nup153 among the known nucleoporins (including Nup153 from nonhuman species) to date (unpublished data). Because Nup124p$^{\Delta AA272-454}$ was part of the Tf1-Gag-binding domain (Balasundaram *et al.*, 1999) and we have shown in this report that Nup124p$^{\Delta AA111-331}$ was required for Tf1-transposition (Figure 2), we reasoned that this domain (Figure 6, B and C, boxes 1–4) could be a critical determinant in the functioning of these two nucleoporins as mediators of Tf1 transposition. We therefore asked if amino acids 272–454 of Nup124p or 448–634 of Nup153 were critical for Tf1 activity. *nmt81-Nup124* (BNB439) expressing the full-length (*WT*) *Nup124* gene was used as the parent plasmid into which all further manipulations were made. In the first instance amino acids 272–454 were deleted in Nup124p (*nup124*$^{\Delta AA272-454}$). Nucleotides encoding the Nup124$^{\Delta AA272-454}$ deletion were cloned into BNB439 to form BNB469. We next replaced Nup124$^{\Delta AA272-454}$ with amino acids 448–634 from Nup153 (*nup124*$^{\Delta AA272-454::Nup153AA448-634}$). Nucleotides encoding the Nup124$^{\Delta AA272-454::Nup153AA448-634}$ fusion were cloned into BNB439 to form BNB470. The *nmt81-nup124*$^{AA1-1159}$, *nmt81-nup124*$^{\Delta AA272-454}$, *and nmt81-nup124*$^{\Delta AA272-454::Nup153AA448-634}$ constructs were expressed in the Δ*nup124* mutant along with a Tf1 reporter plasmid, pHL449–1 (Table 1) to determine the levels of Tf1 cDNA recombination and transposition by genetic assays described earlier under Figure 2, A and B. As depicted in Figure 8A, absence of amino acids 272–454 in Nup124p caused a dramatic fall in Tf1 activity (patches 3–4 comparable to that of the null mutant as negative control, patch 1) when compared with the full-length Nup124p (patch 2), indicating that those amino acids were critical for Tf1 function. However, replacing those deleted amino acids with AA448–634 from the human Nup153 completely restored Tf1 activity (patches 5 and 6) to *WT* levels. These results clearly indicate that the unique domain(s) described above are transferable and therefore, functionally conserved between Nup124p and Nup153. To know if heterologous expression of full-length Nup153 in a Δ*nup124* mutant would reinstate wild-type levels of Tf1 cDNA recombination and transposition, the full-length human *Nup153* gene was cloned from BNB405 into an *S. pombe* vector to form *nmt81:Nup153* $^{AA1-1475}$ (BNB492A/B, Table 2). *nup124* null mutant and *WT* strains bearing these constructs as indicated in Figure 8B were subjected to Tf1 cDNA recombination and transposition assays as previously described. Tf1 cDNA recombination and transposition from strains expressing *nmt81:Nup153* $^{AA1-1475}$ did not complement the Tf1 activity in a Δ*nup124* mutant when compared with an equivalent strain expressing *nmt81:Nup124* $^{AA1-1159}$ (Figure 8B). Taken together, our results demonstrate that expression of Nup153$^{AA448-634}$ within the context of an *nup124*$^{\Delta AA272-454}$ deletion mutation facilitated Tf1 transposition, whereas the full-length *Nup153* is unable to do so in a *nup124* null mutant.

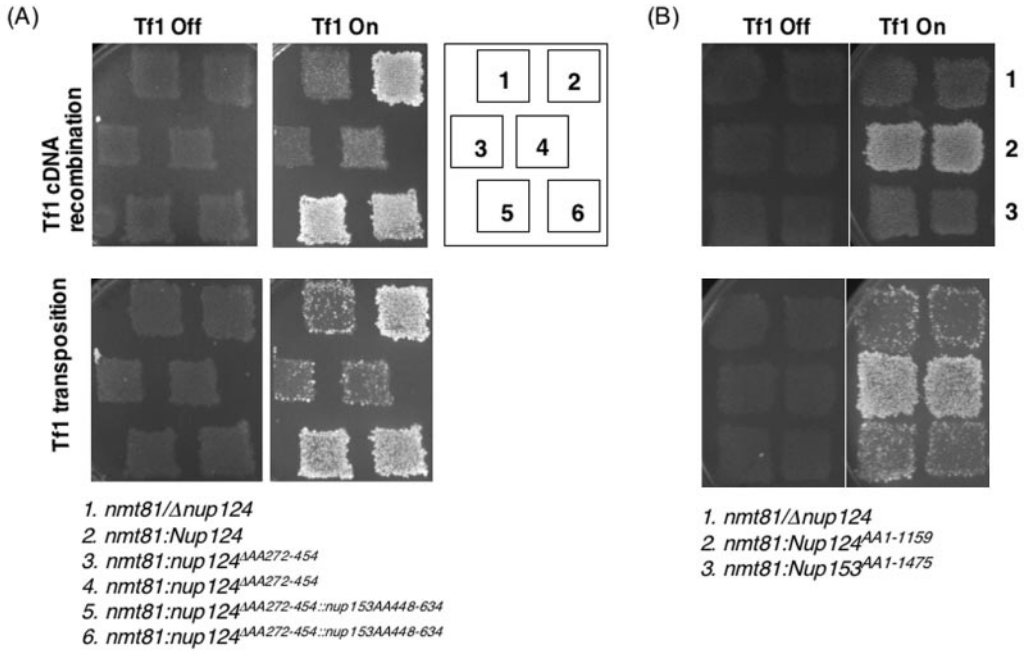

Figure 8. Rescue of Tf1 transposition in a *nup124* mutant by a unique domain of Nup153. (A) Genetic assays to test the efficacy of Tf1 cDNA recombination and transposition were conducted exactly as described earlier in Figure 2 so as to measure the ability of epigenetically expressed mutants (3–6) to reinstate levels of Tf1 cDNA recombination and transposition in a Δ*nup124* mutant (1) compared with epigenetically expressed *Nup124* (2) and the null strain (1). (B) Strains expressing full-length versions of Nup153$^{AA1-1475}$ (YNB964–965), Nup124p$^{AA1-1159}$ (YNB895), and Δ*nup124* (YNB893) were assayed for Tf1 activity as described above.

Abrogation of Tf1 Activity when AA 264–454 of Nup124p or AA447–634 from Human Nup153 Is Overexpressed

Overexpression studies may also be used to ascertain the functionality of a gene or its product if it results in a loss-/gain-of-function or a dominant-negative phenotype. By overexpressing the unique domains of Nup124p and Nup153 in a *WT* background we reasoned that should these domains be interacting with either a viral component or some other factor, then the ratio of interacting components may not be favorable and would result in lowering transposition. To test whether the AA 272–454 of Nup124p or AA448–634 from human Nup153 was mechanistically required for the Tf1 transposition process, we asked if overexpression of the aforementioned fragments would cause an inhibition of Tf1 transposition in a *WT* background *without* inhibiting growth of the host. Assays for dominant negative phenotypes have been successfully used to identify and dissect nuclear transport processes involving two or more interacting species (Iovine *et al.,* 1995; Bastos *et al.,* 1996; Fornerod *et al.,* 1997; Shah and Forbes, 1998) and in this particular context, would be consistent with the titration of a Nup124p-interacting protein by an excess of the Nup124p or Nup153-fragment. Furthermore, such a result would also imply a specific (Tf1-related) function rather than a global effect. Nucleotides encoding Nup124$^{AA264-454}$ and Nup153$^{AA447-634}$ were cloned (AA264–454 and AA447–634 were used instead of AA272–634 and AA448–634, respectively, for purely cloning reasons) into vectors so that their transcription was under the control of either the thiamine repressible *nmt81* (lowest strength) or an *nmt1* promoter (highest strength) (Figure 9B) and expressed in the *WT* along with the Tf1 reporter plasmid, pHL449–1 (Table 1) to determine the levels of Tf1 cDNA recombination and transposition by genetic assays described earlier under Figure 2, A and B. As depicted in Figure 9A, overexpression of both nup124 and nup153 fragments at low levels (*nmt81*, patches 7–8 and 9–10, respectively) did not inhibit Tf1 activity. However, when overexpressed from the highest strength promoter, *nmt1* both fragments caused an equal and almost complete knock-down of Tf1 activity (*nmt1*, patches 11–12 and 13–14, respectively) when compared with the positive (patches 2–6) or negative (patch 1) controls for the Tf1 assay. A 10–20% decrease in colony size was observed between *nmt81*- and *nmt1*-induced cultures when compared with noninduced cultures (Figure 9C), suggesting that full-strength induction of the fragments had a slight to moderate effect on growth. Two conclusions may be drawn from the experiment illustrated in Figure 9. First, overexpression of the domain knocks down Tf1 transposition specifically in a dose-dependent manner without significantly affecting growth and second, the Nup153p$^{AA447-634}$ domain acts in a similar way to the Nup124p$^{AA264-454}$ fragment. Taken together, our data from Figures 8 and 9 demonstrate that a unique domain found in these two nucleoporins Nup124p and Nup153 is essential for Tf1 retrotransposon activity.

DISCUSSION

Role of Nup124p in the Nuclear Import of Tf1-Gag and Tf1 Retrotransposon Activity

In our genetic assay, Tf1 transposition activity was directly related with the ability of cells induced for expression of the Tf1 transposon to integrate copies of its *neo*-marked genome into the host genome thereby rendering the host strain resistant to the drug G418. Tf1 cDNA recombination demonstrated the ability of reverse transcripts of the spliced Tf1 mRNA, if present in the nucleus to homologously recombine with the Tf1-neoAI plasmid and generate a G418 resistant

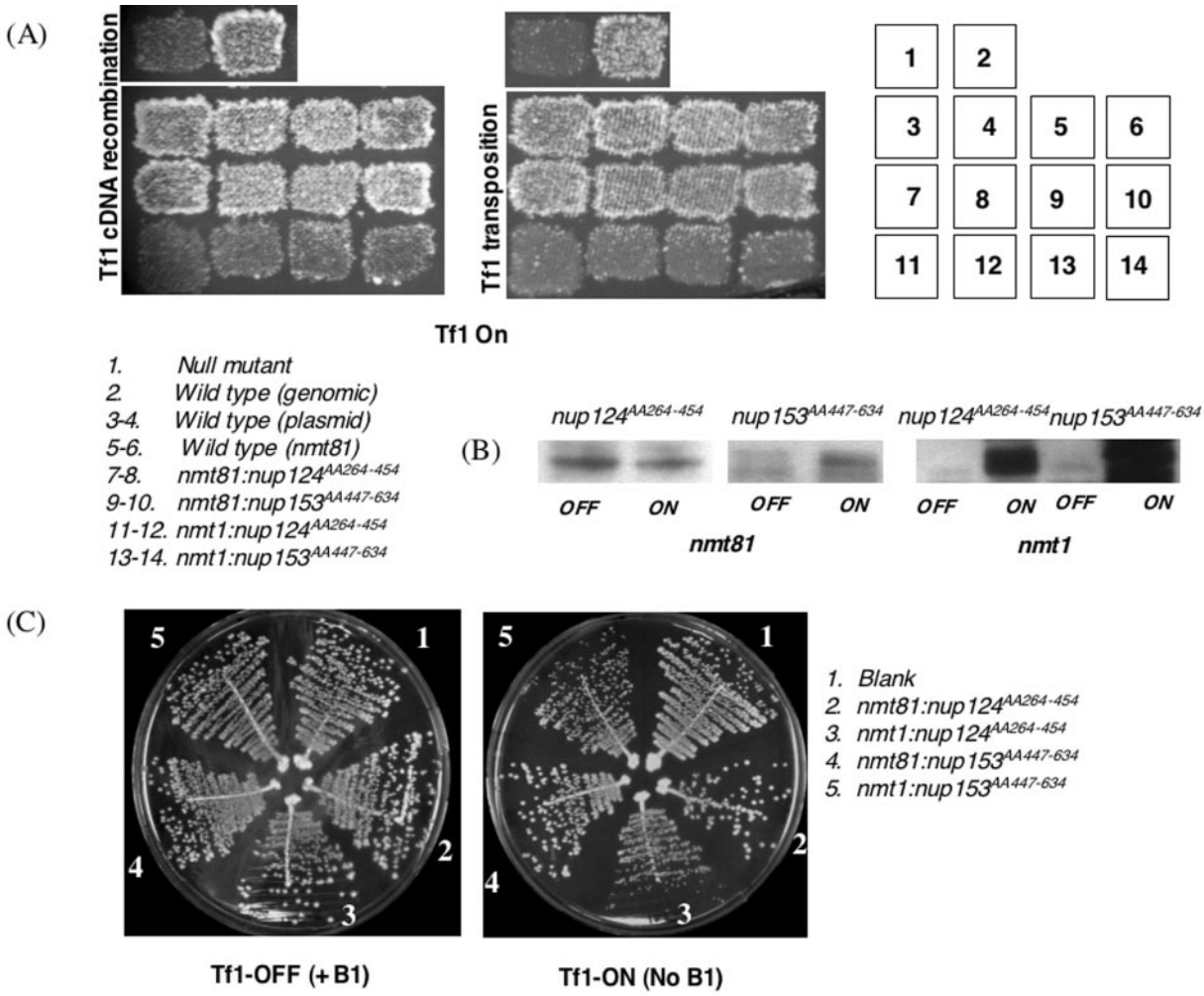

Figure 9. Overexpression of unique domains of Nup124p and Nup153 inhibit Tf1 activity without significantly affecting growth. (A) Multicopy plasmids containing fragments encoding the indicated amino acids from Nup124p and Nup153 whose transcription was under the control of either the *nmt81* (weak) and *nmt1* (strong) promoter were tested in a *WT* strain for Tf1 activity. (1) Null mutant containing an empty vector served as a negative control (YNB893). (2) WT strain expressing genomic *Nup124* and served as a positive control (YNB932). All other strains used are as indicated. (3–4) Multicopy plasmid with *Nup124* expressed from its native promoter in a null mutant background. (5–6, YNB) Multicopy plasmid with *Nup124* expressed from an nmt81 promoter in a null mutant background. (7–8, YNB909–10), (11–12, YNB933–34), and (9–10, YNB911–12), (13–14, YNB935–36) represent Nup124p and Nup153 fragments, respectively, being overexpressed from the indicated promoters. (B) Western blots of nup124$^{AA264-454}$p and nup153$^{AA449-634}$p without (Off) and with (On) induction of *nmt81* and *nmt1* promoters as indicated. Equal volumes of crude extracts from 30 ODs of cells were subjected to PAGE on 8–16% gels. Arrow indicates the protein species recognized by a 1:1000 dilution of anti-V5 HRP (R-961-25, Invitrogen, Carlsbad, CA). (C) The indicated strains were streaked for single colonies onto EMM plates-ura-leu with (Tf1-Off) and without (Tf1-On) thiamine and incubated at 32°C.

version of the plasmid—therefore a measure of efficiency at which Tf1 cDNA may be imported into the nucleus (Levin *et al.*, 1993; Balasundaram *et al.*, 1999). The ability of Tf1-Gag to be imported into the nucleus in these cells correlated well with wild-type levels of Tf1 cDNA recombination and transposition activity. Interestingly, Tf1-Gag appeared exclusively at the NE colocalizing with MAb414-stained FG-nucleoporins even in the absence of Nup124p. It is noteworthy that mere recruitment of Tf1-Gag to the nuclear envelope in the null mutant is not sufficient to promote Tf1 activity implying that Nup124p was not required for recruitment and docking of Tf1-Gag to the NE but rather for translocation of Tf1-Gag into the nucleus. Consistent with the genetic (in vivo) evidence presented here on the involvement of Nup124p in Tf1 transposition is the observation that Nup124p appears to interact with TF1-Gag rather than with SIV-Gag in vitro in our immunoprecipitation assay. On the basis of yeast two-hybrid and GST-pull down assays, we had previously shown that the N-terminal half of Nup124p binds to Tf1-Gag (Balasundaram *et al.*, 1999). Furthermore, the NLS activity of Gag when expressed in a heterologous protein was specifically dependent on the presence of Nup124p (Dang and Levin, 2000). It is not clear, however, whether Nup124p facilitates Tf1-Gag independently or within context of the entire virus-like particle. Tf1-Gag is required for particle assembly in the cytoplasm and is also found associated with 50-nm particles inside the nucleus, implying that Tf1 particles were able to pass through nuclear pores without first being disassembled before nuclear import (Teysset *et al.*, 2003).

Cellular Effects of HIV-1 Vpr Are Mediated via Nup124p

In vivo, Vpr shows multiple activities both in mammalian and *S. pombe* cells, which include nuclear transport, induction of cell cycle G2 arrest, morphological changes, and cell death (Di Marzio *et al.*, 1995; Rogel *et al.*, 1995; Elder *et al.*, 2000, 2002; de Noronha *et al.*, 2001; Chang *et al.*, 2004). Several lines of evidence presented here suggest that nuclear import of HIV-1 Vpr in the fission yeast involves Nup124p. Our investigations show that absence of Nup124p gives cells expressing Vpr a distinct growth advantage in contrast to wild-type cells. Indeed, such an observation is strong evidence in favor of a critical role for Nup124p in Vpr-mediated cell toxicity. Second, presence or absence of Nup124p affects the nuclear localization of HIV-1 Vpr. Finally, Nup124p appears to interact weakly with Tf1-Gag or HIV-1 Vpr in an in vitro coimmunoprecipitation assay. To eliminate the possibility of artifacts in immunoprecipitation arising from the use of a single antibody, we tested the ability of both proteins to be recovered from immunoprecipitates in any given set of reactions independently by two different antibodies. Despite numerous attempts, we were unable to secure stoichiometric amounts of both sets of proteins in our immunoprecipitates. Three possibilities may be entertained. One is that the interaction between Nup124p and Tf1-Gag or Vpr is weak or transient. The other is because our interaction data were derived from in vitro reactions, it is possible that some putative cofactors in the reticulocyte lysate could be different from those found in the fission yeast. Finally, that our observed interactions may not be direct. It is possible that Nup124p is part of a subcomplex at the NPC and that we may be missing additional elements or yeast specific factors not present in the reticulocyte lysate that may in fact, contribute to binding at the pore.

Because a significant fraction of Vpr could interact with components of NPC (Fouchier *et al.*, 1998; Popov *et al.*, 1998a, 1998b; Fouchier and Malim, 1999; Le Rouzic *et al.*, 2002), it is possible that Vpr needs to localize to the interior of the nuclear rim or to associate with certain proteins at the nuclear face of the nuclear envelope to be effective in its cell killing activity. We had previously observed that Nup124p concentrated within the region between the nuclear and cytoplasmic faces of the NPC (Balasundaram *et al.*, 1999). Thus, in the absence of Nup124p from the NPC, Vpr may be denied access to nuclear components it would otherwise interact with. The ability of Vpr to disrupt normal nuclear morphology was noted in previous studies in *S. pombe* (Zhao *et al.*, 1998; Chang *et al.*, 2004). Recently, Chang *et al.* (2004) have observed defects in the assembly and function of the mitotic spindle in *S. pombe* expressing HIV-1 Vpr. Their results suggested that perturbations in nuclear architecture, in fact, might lead to delocalization of two proteins sad1p and the polo kinase plo1p from the spindle pole body (SPB), resulting in defects in the assembly and function of the mitotic spindle. de Noronha *et al.* (2001) observed that Vpr expression induced transient herniations or blebbing in the nuclear lamina, leading to local bursting and mixing of nuclear and cytoplasmic components, especially some key cell cycle regulators like Cdc25, cyclin B1, Wee 1, and perhaps many other soluble components. Although yeast nuclei do not possess a nuclear lamina, these NE herniations or bleb-like structures are clearly visible in wild-type cells of *S. pombe* when Vpr is expressed (depicted in Figure 4 and Y. Zhao, personal communication), suggesting a common mechanism that underlies Vpr activity in both mammalian systems and yeast.

Is the Vertebrate (human) Nup153p a Functional Homolog of the Fission Yeast Nup124p?

Proteomic comparisons of nucleoporins between yeast (*S. cerevisiae*) and mammals reveal a moderate level of conservation despite anatomical differences and a very low sequence homology (Rout *et al.*, 2000; Cronshaw *et al.*, 2002). However, the extent of functional homology has yet to be fully realized. In general, sequence comparisons between nucleoporins from mammalian and yeast (*S. cerevisiae*) yield very poor alignments and in many cases direct sequence homology is not apparent or even feasible. The mammalian Nup153 and its possible homologues in yeast is a case in point with some reports implying that the vertebrate Nup153 has no identifiable yeast homolog (Vasu *et al.*, 2001; Walther *et al.*, 2001), whereas others recording Nup1p of *S. cerevisiae* as its homolog (Stoffler *et al.*, 1999; Cronshaw *et al.*, 2002). Similarly, Nup60p of *S. cerevisiae* was considered a yeast "version" of Nup153 based on common binding partners rather than comprehensive sequence homology (Hase and Cordes, 2003). Our own analysis summarized in Figure 6 shows that Nup124p may be a possible homolog of Nup153 based on sequence comparison. We therefore reasoned that in a manner analogous to that of Nup124p, the mammalian Nucleoporin Nup153p might be the responsible for translocating Vpr into the nucleus. Nup153, like Nup124p, binds weakly to both Vpr and Tf1-Gag. Furthermore, both nucleoporins did not bind SIV-Gag or SIV-Vpx, indicating that their interaction with Tf1-Gag was selective, whereas they exhibited a very weak binding to SIV-Vpr. The latter result is possible given the high sequence conservation between Vpr from HIV-1 and SIV isolates (see Figure 1 in Zhou and Ratner, 2000). However, expression of full-length Nup153 failed to complement the Tf1 transposition defect in a *nup124* null mutant. An immediate admission to our findings reported here therefore is that the vertebrate Nup153 is not a homolog of Nup124p. Yet, swapping the fission yeast *nup124* gene fragment (encoding $AA^{272-454}$) with the *nup153* gene fragment (encoding $AA^{448-634}$) was indeed able to restore wild-type levels of Tf1 cDNA recombination and transposition in a $nup124p^{\Delta AA272-454}$ mutant. Furthermore, the ability of $Nup124p^{AA264-454}$ and $Nup153AA^{447-634}$ to similarly cause a dose-dependent inhibition of the Tf1 cDNA recombination and transposition processes (specifically inhibiting viral replication by overexpression of critical nucleoporin domains is an attractive antiviral strategy) may be seen as additional evidence in support of that domain in Nup153 as being the functional homolog of the respective domain in Nup124p. $AA^{448-634}$ of huNup153 is devoid of any recognizable motif (Sukegawa and Blobel, 1993; McMorrow *et al.*, 1994), whereas $Nup124p^{AA272-454}$ fell within the domain required for Tf1-Gag binding (Balasundaram *et al.*, 1999) and the $nup124p^{\Delta AA111-333}$ mutant did not support Tf1 activity (see Figure 2B). Thus, in the context of our results, Nup153 may have evolutionarily retained one Tf1-transposition competent domain and lost one (or more) other required domain(s) or conversely, acquired other specialized domains that may, within the context of the full-length protein, inhibit Tf1 transposition activity. In the latter scenario, multiple domains unique to Nup153 do not appear to have apparent equivalents in Nup124p (or in Nup1p for that matter). These domains are the TRN1-binding domain or M9 NLS, $AA^{235-300}$ (Nakielny *et al.*, 1999), the RNA-binding domain, $AA^{250-400}$ (Dimaano *et al.*, 2001), and the Zn-finger domain, $AA^{657-880}$ (Sukegawa and Blobel, 1993; McMorrow *et al.*, 1994).

The presence of short stretches of conserved amino acids between Nup124p and huNup153 might indicate that both proteins derive from a common ancestor. This is perhaps equally true of Nup60 (Hase and Cordes, 2003) or Nup1p (Stoffler *et al.*, 1999; Cronshaw *et al.*, 2002). Nup153 having acquired additional domains concomitant with its involvement in specialized tasks like in nuclear envelope breakdown and reformation during cell division (Bodoor *et al.*, 1999; Daigle *et al.*, 2001) through its interaction with lamin B_3 (Smythe *et al.*, 2000), the COP1 complex (Liu *et al.*, 2003), and ability to anchor other proteins at the NPC (Walther *et al.*, 2001). In the fission yeast, such a role for Nup124p is expected to be redundant since there is no "open" mitosis. Furthermore, in vertebrate cells, Nup153 is believed to play key roles in import of proteins into the nucleus (Radu *et al.*, 1995; Shah and Forbes, 1998; Shah *et al.*, 1998), export of proteins and RNA from the nucleus (Bastos *et al.*, 1996; Ullman *et al.*, 1999; Ball *et al.*, 2004), and in the architecture, assembly, and functioning of the NPC (Nakielny *et al.*, 1999; Smythe *et al.*, 2000; Walther *et al.*, 2001; Fahrenkrog *et al.*, 2002; Hutchison, 2002; Griffis *et al.*, 2004). In the fission yeast, such essential tasks may be distributed among other nucleoporins because Nup124p may be deleted without loss of nuclear transport function or viability and to the best of our knowledge has no other phenotype other than the Tf1 transposition defect (Balasundaram *et al.*, 1999).

Future Perspectives

Nup153p is described to have close structural and functional associations with the nuclear lamina (Smythe *et al.*, 2000), the site of Vpr-induced NE herniations or blebs and showing significant changes in patterns of lamins A, B and C expression (de Noronha *et al.*, 2001). A direct interaction between Vpr and lamins was sought but not detected (de Noronha *et al.*, 2001). Though the mechanism behind the formation and bursting of Vpr-induced blebs remains unknown (de Noronha *et al.*, 2001), it is tempting to speculate that Nup153 may be one of the key components affected, given its location on the distal nucleoplasmic ring of the NPC (Pante *et al.*, 1994) and its association with lamin B_3 (Smythe *et al.*, 2000). Recent work in *S. cerevisiae* and *S. pombe* has identified and described the presence of nucleoplasmic rings, basket or fishtrap, and other NPC structures similar to those found in higher eukaryotic species (Kiseleva *et al.*, 2004). Although yeasts do not possess a lamin structure undergirding the nuclear envelope, it is proposed that there may be equivalent structures and components including the yeast equivalent of Nup153 (Hutchison, 2002). Because in our studies, Nup153, like Nup124p was able to bind HIV-1 Vpr in vitro and the overexpression of the $Nup153AA^{447-634}$ domain was able to inhibit replication of the Tf1 retrotransposon, our observation of the Vpr-induced blebs and death in wild-type cells of *S. pombe* may be consistent with a similar pathology found in mammalian cells. It still, however, remains to be determined if overexpression of $Nup153AA^{448-634}$ in HeLa cells will prevent the Vpr-induced nuclear envelope blebbing or inhibit HIV-1 infection of macrophages. Another exciting prospect is to find binding partners of Nup124p or Nup153 or Nup124p/Nup153 chimeras required for Tf1 transposition or HIV-1 Vpr activity in the fission yeast. It is likely that these binding partners in the fission yeast will lead to identification of proteins with corresponding functions in mammalian systems that affect nuclear import of viral components. The recent availability of mutants for nucleoporins and nuclear transport factors as those described for budding yeast (Strawn *et al.*, 2004) or fission yeast (Chen *et al.*, 2004) will serve as powerful tools to understand the mechanism of nuclear import and action of viruses or viral components that do require an obligatory nuclear passage.

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