Multiple Conserved Domains of the Nucleoporin Nup124p and Its Orthologs Nup1p and Nup153 Are Critical for Nuclear Import and Activity of the Fission Yeast Tf1 Retrotransposon[□]

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Submitted December 1, 2006; Revised June 18, 2007; Accepted June 26, 2007

Monitoring Editor: Susan Wente

The nucleoporin Nup124p is a host protein required for the nuclear import of both, retrotransposon Tf1-Gag as well as the retroviral HIV-1 Vpr in fission yeast. The human nucleoporin Nup153 and the *Saccharomyces cerevisiae* Nup1p were identified as orthologs of Nup124p. In this study, we show that all three nucleoporins share a large FG/FXFG-repeat domain and a C-terminal peptide sequence, GRKlxxxxRRKx, that are absolutely essential for Tf1 retrotransposition. Though the FXFG domain was essential, the FXFG repeats themselves could be eliminated without loss of retrotransposon activity, suggesting the existence of a common element unrelated to FG/FXFG motifs. The Nup124p C-terminal peptide, GRKlAVPRSRKR, was extremely sensitive to certain single amino acid changes within stretches of the basic residues. On the basis of our comparative study of Nup124p, Nup1p, and Nup153 domains, we have developed peptides that specifically knockdown retrotransposon activity by disengaging the Tf1-Gag from its host nuclear transport machinery without any harmful consequence to the host itself. Our results imply that those domains challenged a specific pathway affecting Tf1 transposition. Although full-length Nup1p or Nup153 does not complement Nup124p, the functionality of their conserved domains with reference to Tf1 activity suggests that these three proteins evolved from a common ancestor.

INTRODUCTION

The propagation of Tf1, a long terminal repeat (LTR)-containing retrotransposon in the fission yeast *Schizosaccharomyces pombe* (Levin *et al.*, 1990; Levin and Boeke, 1992) is known to require many of the same processes used by retroviruses to complete its life cycle within the host cell. Yeast retrotransposons can therefore serve as very useful models for the replication of retroviruses (Levin *et al.*, 1993; Irwin *et al.*, 2005; Kelly and Levin, 2005). The full-length transcript of the Tf1 retroelement encodes gag, protease (PR), reverse transcriptase (RT), and integrase (IN) proteins that assemble along with copies of RNA to form 50-nm virus-like particles (VLPs; Levin *et al.*, 1993; Haag *et al.*, 2000; Teyssset *et al.*, 2003). To integrate its genome into the target cell, it is assumed that Tf1-VLPs move to the nucleus and are transported through the central channel of the nuclear pore complex (NPC). In fact, Tf1 particles were observed within the nucleus associated with heterochromatin (Teyssset *et al.*, 2003). Though details of the nuclear import mechanism remain unclear, there is substantial evidence that both Tf1-Gag and the NPC nucleoporin Nup124p are key determinants of this process (Atwood *et al.*, 1996; Balasundaram *et al.*, 1999;

Dang and Levin, 2000; Teyssset *et al.*, 2003; Kim *et al.*, 2005; Varadarajan *et al.*, 2005). Similarly, NPC elements (including nucleoporins) governing the nuclear entry of the retrotransposon Ty3 cDNA in *Saccharomyces cerevisiae* had either a positive or negative impact on its replication (transposition), implying the crucial role of the nuclear pore in regulating retroviral activity (Irwin *et al.*, 2005). Although an NLS required for nuclear entry of Ty1 and Ty3 was identified within the C-terminal domain of their respective integrases (Kenna *et al.*, 1998; Lin *et al.*, 2001), a possible interaction of Gag3 with the NPC was suggested with Ty3 (Irwin *et al.*, 2005).

The NPC is a large structure embedded in the double membrane of the nuclear envelope, with a molecular weight of approximately 125 MDa in vertebrates and 66 MDa in yeast (Fahrenkrog and Aebi, 2003; Antonin and Mattaj, 2005). Despite differences in both size and composition, yeast and vertebrate NPCs have a number of common features including their general overall structure, protein composition, and mechanism of nuclear transport (Fabre and Hurt, 1997; Yang *et al.*, 1998; Stoffler *et al.*, 1999; Rout *et al.*, 2000; Ryan and Wentz, 2000; Wentz, 2000; Cronshaw *et al.*, 2002; Suntharalingam and Wentz, 2003; Kiseleva *et al.*, 2004; Devos *et al.*, 2006; Tran and Wentz, 2006). Fission yeast has NPC features and transport factors comparable to *S. cerevisiae* and vertebrate systems and the appropriate tools to study these features are now available (Ding *et al.*, 2000; Chen *et al.*, 2004; Kiseleva *et al.*, 2004; Yoshida and Sazer, 2004). In general, the bulk of macromolecular traffic through the NPC is mediated by a system of mobile transporter proteins, multiple importins α and β and the nucleoporins (Nups), the latter, a unique and conserved group of structural proteins of the pore complex. The most highly con-

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E06-12-1062>) on July 5, 2007.

[□] The online version of this article contains supplemental material at *MBC Online* (<http://www.molbiolcell.org>).

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served feature between yeast and mammalian nucleoporins is a dipeptide repeat motif, in the form phenylalanine-glycine (FG), glycine-leucine-phenylalanine-glycine (GLFG), or phenylalanine-X (any amino acid)-phenylalanine-glycine (FXFG), present in approximately one-third of all core components of the pore (Rout and Aitchison, 2000; Rout *et al.*, 2000; Cronshaw *et al.*, 2002). Many NPC-binding sites for importins/exportins and NTF2 carriers are supplied by these FG-repeat-containing Nups (Ryan and Wentz, 2000). Although the presence of FG repeats is a feature shared by many Nups, the intervening sequences between FG repeats differ drastically among them. What contribution, if any, the surrounding amino acids make to the FG-binding sites is unclear (Cushman *et al.*, 2006). One possibility is that FG-Nups may form a meshwork within the central channel of the pore where they are involved in translocation (Ribbeck and Gorlich, 2001). However, the essential functionality of the FG/FXFG/GLFG repeats in Nups is in question because most asymmetrically localized Nups (either to the cytoplasmic or nucleoplasmic faces of the NPC) may be eliminated without loss of viability (Strawn *et al.*, 2004). FG/GLFG/FXFG-repeat-containing Nups are known to bind HEAT repeats on importin β (Bayliss *et al.*, 2002b). Kap95, an importin β , is an essential protein (Chen *et al.*, 2004), and there are two importin α type 1, Imp1p and Cut15p, both of which have partially overlapping and distinct functions in *S. pombe* (Umeda *et al.*, 2005). Both Cut15p and Imp1p were able to import classical NLS-containing substrates in vivo and in vitro into the nucleus, implying that each is an authentic importin α with possibly common binding partners (Umeda *et al.*, 2005). There is compelling in vivo evidence that interaction between importin α and FG Nups may be the basis for the latter to either concentrate carrier-cargo complexes at the NPC entrance and/or participate in a sequence of docking and undocking interactions as the carrier-cargo complexes transit through NPCs (Stewart *et al.*, 2001).

We had previously demonstrated that the fission yeast Nup124p, a nonessential FG repeat-containing nucleoporin was a host factor required for Tf1 transposition (Balasundaram *et al.*, 1999; Varadarajan *et al.*, 2005). We also predicted that such a function was a unique one, because no housekeeping functions were disrupted in the absence of Nup124p (Balasundaram *et al.*, 1999). Indeed, nuclear import of Tf1-Gag was impeded, and Tf1 transposition was markedly reduced in the *nup124* null mutant (Balasundaram *et al.*, 1999; Varadarajan *et al.*, 2005), despite the presence of at least eight other FG-nucleoporins (Denning and Rexach, 2007). Furthermore, the N-terminus contains a putative Tf1-Gag-binding domain and the C-terminus of Nup124p contains the FXFG domain essential for retrotransposition but not for localization of either Nup124p or Tf1-Gag to the NPC, reiterating the fact that Nup124p possessed special features required for the nuclear import/translocation of the retrotransposon Tf1 (Tf1-Gag; Balasundaram *et al.*, 1999; Dang and Levin, 2000; Varadarajan *et al.*, 2005). In this report, we have investigated the mechanism of Nup124p-mediated Tf1 import into the nucleus. We have studied the regions at the C-terminus of Nup124p required for Tf1 transposition by mapping two key domains, the FXFG-repeat domain and a well-conserved C-terminal peptide, and identified key residues within a well-conserved C-terminal peptide. We present evidence to show that though the FXFG repeats themselves are redundant, the FXFG domain and a conserved C-terminal motif GRKlxxxxRRKx of Nup124p and those of its orthologs, Nup1p and Nup153, play a critical and unique role in Tf1 activity. We also show that Nup124p interacts with key components of the fission yeast nuclear transport system, Imp1p and Cut15p (importin α) and Kap95 (importin β), in addition to

Tf1-Gag. Finally, we demonstrate that overexpressing the conserved domains of Nup124p selectively knocks down Tf1 activity by preventing Tf1-Gag from being imported into the nucleus without affecting the growth of the host cell, making elements of the nuclear pore complex an attractive option for the development of antiviral strategies.

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 his3Δ nup124::HIS3 ade6-M216* and YNB16, *h-leu1-32 ura4-294* were the principal yeast strains used in this study and are referred to in the text as null mutant ($\Delta nup124$) and wild type (WT), respectively. YNB19 and YNB16 were transformed with various plasmids described in Table 2 (see also Supplementary Table S1). Transformants were routinely propagated in EMM –ura –leu + 15 μ M thiamine. When expression of the desired sequence was required, strains were grown in EMM –ura –leu without thiamine to induce the *nmt1* (strong) or *nmt81* (weak) promoter (*nmt*, no message in thiamine).

Plasmid Constructions

DNA fragments used to create plasmids for this study (Supplementary Table S1) were generated by PCR. All constructs were confirmed by DNA sequencing. Because many of the constructs were of a complex nature, a simplified naming system was adopted and used throughout this work (Table 2), unless otherwise specified.

Tf1 Transposition and Tf1 cDNA Recombination Assays

Tf1 transposition and Tf1 cDNA recombination were assayed as previously described (Balasundaram *et al.*, 1999; Varadarajan *et al.*, 2005). Briefly, Tf1 transposition was monitored by placing a *neo*-marked Tf1 element under the control of the inducible *nmt1* promoter. The bacterial *neo* gene allowed cells to grow in the presence of 500 μ g of G418/ml, and thus Tf1 transposition activity could be correlated with the ability of cells to grow on G418-containing medium. *S. pombe* strains that contained a Tf1-*neo* plasmid (pHL449-1) were grown as patches on EMM –ura dropout agar plates in the presence of thiamine (Tf1 Off) or in the absence of thiamine (Tf1 On). The transcription of the *nmt1*-Tf1-*neo* gene is induced in the absence of thiamine (Tf1 On). After 4 d of incubation at 32°C, these plates were replica printed to medium containing 5-fluoroorotic acid (5-FOA; 100 mg/ml) to eliminate the *URA3*-Tf1-*neo* plasmid. Finally, 5-FOA⁺ patches were printed to medium containing both 5-FOA and G418 and incubated at 32°C for 38 h to detect strains that became resistant to G418 as the result of insertions of the *neo*-marked Tf1 element into the genome. The presence of Tf1 cDNA in the nucleus was examined by cDNA recombination assays. This protocol is similar to that of the transposition assay in that strains with the *neoAI*-marked Tf1 plasmid 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 YES medium that contained 500 μ g of G418/ml. Recombination between cDNA and cellular transposon sequences was scored on the G418 plates after 38 h of growth at 32°C. Because the main goal of this study was to examine the role(s) of nuclear pore/transport components and their ability to import Tf1 components into the nucleus, only cDNA recombination assays are depicted and referred to throughout the text and in the figures as “Tf1 Activity.”

Fluorescence Microscopy

Cells totaling ~1.0 OD₆₀₀ (optical density at 600 nm) were harvested and fixed in 3.7% formaldehyde in PBS for 5 min at room temperature. Fixed cells were washed twice in 1× phosphate-buffered saline with 0.1% Triton-X and mounted onto glass slides with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA). Laser-scanning confocal microscopy was performed using an Olympus upright Confocal microscope (Fluoview; Melville, NY), with red fluorescent protein (RFP) visualized using the Cy3 laser (543 nm) and yellow fluorescent protein (YFP) using the enhanced green fluorescent protein (GFP) laser (488 nm). DAPI was visualized using the DAPI laser at 405 nm. For indirect immunofluorescence, cells were harvested at 0.4–0.8 OD₆₀₀ and treated as previously described (Balasundaram *et al.*, 1999; Varadarajan *et al.*, 2005). Hemagglutinin epitope (HA)-tagged proteins were visualized with anti-hemagglutinin antibody (clone 12CA5, Roche Biochemicals, Basel, Switzerland) at a dilution of 1:1000. 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 containing DAPI (Vector Laboratories). Epifluorescence microscopy was performed at 1000× magnification using a Leica DMLB microscope (Deerfield, IL) equipped with an Optronics DEI-750T coded CCD camera (Optronics, Goleta, CA). Leica Qwin proprietary software was used to capture images. All images were processed for figure presentation using Adobe Photoshop 7.0 software.

Table 1. Yeast strains

Strain (YNB)	Parent strain/source	Plasmid			
		Ura ⁺		Leu ⁺	
		No.	Character	No.	Character
10	YHL 4990	pHL490	PROT ^{fs} (frameshift mutation in Tf1-protease)	pSP1	Empty vector
11	YHL 4992	pHL476	In ^{fs} (frameshift mutation in Tf1-integrasen)	pSP1	Empty vector
16	YHL 912	—	—	—	—
19	YHL 7143	—	—	—	—
58	YNB 19	pHL449-1	Tf1 reporter: neoAI-marked version of <i>nmt1:Tf1</i> in an <i>ura4</i> selectable marker	pBNB 12	3HA:Nup124
59	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 31	3HA:nup124ΔAA571-615
65	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 21	3HA:nup124ΔAA1089-1111
235	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 103	3HA:nup124ΔAA828-967
236	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 105	3HA:nup124ΔAA998-1026
350	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 136	3HA:nup124 R1154A
352	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 139	3HA:nup124 R1156A
354	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 142	3HA:nup124 R1157A
356	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 148	3HA:nup124 K1158A
357	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 149	3HA:nup124 R1159A
549	YNB 19	BNB 193	Tf1-Gag:YFP	pBNB 12	3HA:nup124
550	YNB 19	BNB 193	Tf1-Gag:YFP	pBNB 155	3HA:nup124AA1154RSRRKR1159- ASAAAA
552	YNB 19	BNB 193	Tf1-Gag:YFP	pBNB 93	3HA:nup124-1
553	YNB 19	BNB 193	Tf1-Gag:YFP	pBNB 117	3HA:nup124ΔAA571-1111
554	YNB 19	BNB 193	Tf1-Gag:YFP	pBNB 119	3HA:nup124Δ111-331
893	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB418	<i>nmt81</i> :empty vector
967	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 492	<i>nmt81</i> :nup153
1017	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 572	<i>nmt81</i> :nup1
1018	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 524	3HA:nup124 G1147S
1020	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 526	3HA:nup124 R1148A
1028	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 552	3HA:nup124ΔAA1151-1155: nup153AA1468-1472
1030	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 554	3HA:nup124ΔAA1156-1159: nup153AA1473-75
1032	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 556	3HA:nup124ΔAA1151-1155
1034	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 558	3HA:nup124ΔAA1156-57
1040	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 531	3HA:nup124 I1150A
1042	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 532	3HA:nup124Δ1147-1150
1044	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 541	3HA:nup124 K1158A R1159A
1046	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 542	3HA:nup124 AA1157-59A
1048	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 544	3HA:nup124 AA1156-59A
1050	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 533	3HA:nup124 ΔAA 1147-1159
1052	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 535	3HA:nup124ΔAA 571-1146
1054	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 539	3HA:nup124 ΔAA1147-1159: nup153 AA 1464-1475
1058	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 550	3HA:nup124 ΔAA1147-1159: nup1AA1065-1076
1062	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 601	3HA:nup124 ΔAA1147-1159: nup1AA1065-1076 +R
1064	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 573	<i>nmt81</i> :nup124
1066	YNB 16	pHL449-1	Tf1-reporter (see above)	pBNB 579	<i>nmt1</i> :GFP:GRK1AVPSRRKR
1070	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 610	3HA:nup124 AA1156-57AA
1086	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 623	<i>nmt81</i> :nup124Δ AA828-967
1096	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 652	<i>nmt81</i> : nup124 ΔAA570-1146: nup153 AA 903-1463
1098	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 664	<i>nmt81</i> :nup1AA1-1076+R
1117	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 672	<i>nmt81</i> :nup124 ΔAA571-1146: nup1 AA 406-1065
1121	YNB 16	pHL449-1	Tf1-reporter (see above)	pBNB 647	<i>nmt1</i> :nup124 AA 571-1111
1125	YNB 16	pHL449-1	Tf1-reporter (see above)	pBNB 649	<i>nmt1</i> :nup153 AA 903-1418
1140	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 640	Nup124ΔAA828-967+ AA571-574, 612-615, 998-1001 , 1023-026, 1089-1092, 1108-1111-AAAA
1141	YNB 16	pHL449-1	Tf1-reporter (see above)	pBNB 696	<i>nmt1</i> :nup1 AA 406-988

Continued

Table 1. Continued

Strain (YNB)	Parent strain/source	Plasmid			
		Ura ⁺		Leu ⁺	
		No.	Character	No.	Character
1189	YNB 16	BNB 271	pREP82	pBNB 736	<i>nmt1</i> :RFP:YAHSDATMVCMFS
1191	YNB 16	BNB 193	Tf1-Gag:YFP	pBNB 736	<i>nmt1</i> :RFP:YAHSDATMVCMFS
1197	YNB 16	BNB 271	pREP82	pBNB 671	<i>nmt1</i> :RFP:GRKIAVPSRRKR
1198	YNB 16	BNB 193	Tf1-Gag:YFP	pBNB 671	<i>nmt1</i> :RFP:GRKIAVPSRRKR
1201	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 725	<i>nmt81</i> : nup124ΔAA571-1146:nup120 AA517-1137
1205	YNB 16	pHL449-1	Tf1-reporter (see above)	pBNB 699	<i>nmt1</i> :nup120AA 517-1137
1209	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 527	3HA:nup124 K1149A
1211	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 546	3HA:nup124 AA1155-59A
1213	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 548	3HA:nup124 AA1154-59A
1215	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 577	3HA:nup124 ΔAA1147-1159:nup153 AA 1464-1475 + R
1217	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 564	<i>nmt81</i> :nup153 Δ AA1468-1475: nup124 AA 1147-1159
1219	YNB 16	pHL449-1	Tf1-reporter (see above)	pBNB 736	<i>nmt1</i> :RFP:YAHSDATMVCMFS
1221	YNB 16	BNB 193	Tf1-Gag:YFP	pBNB 776	<i>nmt1</i> :RFP:nup120AA517-1137
1223	YNB 16	BNB 193	Tf1-Gag:YFP	pBNB 778	<i>nmt1</i> :RFP:nup124 AA571-1111
1227	YNB 16	—	—	pBNB 773	<i>nmt1</i> :GRKIAVPSRRKR:GFP-LacZ
1205	YNB 16	pHL449-1	Tf1-reporter (see above)	pBNB 699	<i>nmt1</i> :nup120AA 517-1137
1209	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 527	3HA:nup124 K1149A
1211	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 546	3HA:nup124 AA1155-59A
1213	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 548	3HA:nup124 AA1154-59A
1215	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 577	3HA:nup124 ΔAA1147-1159:nup153 AA 1464-1475 + R
1217	YNB 19	pHL449-1	Tf1-reporter (see above)	pBNB 564	<i>nmt81</i> :nup153 Δ AA1468-1475: nup124 AA 1147-1159
1219	YNB 16	pHL449-1	Tf1-reporter (see above)	pBNB 736	<i>nmt1</i> :RFP:YAHSDATMVCMFS
1221	YNB 16	BNB 193	Tf1-Gag:YFP	pBNB 776	<i>nmt1</i> :RFP:nup120AA517-1137
1223	YNB 16	BNB 193	Tf1-Gag:YFP	pBNB 778	<i>nmt1</i> :RFP:nup124 AA571-1111
1227	YNB 16	—	—	pBNB 773	<i>nmt1</i> :GRKIAVPSRRKR:GFP-LacZ
1230	YNB 16	BNB 271	pREP82	pBNB 776	<i>nmt1</i> :RFP:nup120AA517-1137
1232	YNB 16	BNB 271	pREP82	pBNB 778	<i>nmt1</i> :RFP:nup124 AA571-1111
1234	YNB 16	BNB 193	Tf1-Gag:YFP	pBNB 418	<i>nmt81</i> :empty vector
1253	YNB 16	pHL449-1	Tf1-reporter (see above)	pBNB 8	<i>nmt1</i> :GFP

Coimmunoprecipitation and In Vitro Binding Assay

All incubations were carried out at 4°C unless otherwise noted. Nup124p, Tf1-Gag, Kap95, Cut15p, Imp1p, Grn1p, and HIV-1 Gag proteins were synthesized using a TNT-coupled reticulocyte lysate system (Promega, Madison, WI), according to the manufacturer's instructions. Equal amounts of in vitro-translated proteins were mixed and incubated for 60 min at 4°C in binding buffer (25 mM HEPES, pH 7.9, 150 mM KCl, 0.1% Nonidet P-40, 5% glycerol, 0.5 mM dithiothreitol, and 0.4 mM phenyl methyl sulfonyl fluoride). Respective antibodies were added to each tube and incubated for 90 min at 4°C. Protein A/G-agarose (Santa Cruz Biotechnology, Santa Cruz, CA; 5 mg per tube) was added to all the tubes, which were then incubated overnight on a nutator. The beads were washed three times with the binding buffer. The immunoprecipitated protein complexes were eluted from the Agarose beads and subjected to SDS/PAGE in 4-15% gels. The gels were processed for fluorography.

RESULTS

The Nup124p FXFG-Repeats Are Not Required for Tf1 Transposition

We had previously reported that the 550-amino acid long 11-FXFG-repeat domain of Nup124p was absolutely required for Tf1 transposition and that the absence of retrotransposition activity in mutants lacking the FXFG domain was associated with the inability of Tf1-Gag to be localized in the nucleus (Balasundaram *et al.*, 1999; Varadarajan *et al.*, 2005). In an attempt to identify whether all of the 11 or

certain crucial FXFG repeats were required for function, various deletions of the FXFG domain were created as indicated in Figure 1 (see Table 2 for details) and expressed in *S. pombe* along with a Tf1 reporter plasmid, pHL449-1 to determine the levels of Tf1 cDNA recombination and transposition (Tf1 activity) by in vivo complementation of a null mutant using published genetic assays (Balasundaram *et al.*, 1999; Varadarajan *et al.*, 2005) as described in *Materials and Methods*. As seen in Figure 1 (see Table 2 for details), although the entire FXFG-repeat-containing region (patches 17-18) was required for Tf1 activity, all the FXFG-domain deletion mutants tested (patches 5-14) yielded wild-type Tf1 activity. Such a result implied that the subdomains of FXFG domain were redundant and that one subdomain could compensate for another. We therefore used a nup124 mutant tagged with three copies of the HA epitope at its N-terminus, 3HA:nup124 ΔFXFG 3-7 as the parent, to systematically and sequentially mutate the remaining FXFG repeats to stretches of alanine quadruples (AAAA). Each of the mutants generated was tested for its ability to support Tf1 transposition (only the mutant Nup124ΔFXFG 3-7, FXFG 1-2, 8-11 → AAAA (see Table 2 for details) is shown in patches 15 and 16). As can be seen from Figure 1, patches 15 and 16, mutation of the FXFG repeats did not have any effect

Table 2. Construct details

Construct	Details
3HA:nup124ΔFXFG1-2	Nup124 ^{ΔAA571-615}
3HA:nup124ΔFXFG3-7	Nup124 ^{ΔAA828-967}
3HA:nup124ΔFXFG8-9	Nup124 ^{ΔAA998-1026}
3HA:nup124ΔFXFG10-11	Nup124 ^{ΔAA1089-1111}
3HA:nup124ΔFXFG3-7*	Nup124 ^{ΔAA828-967}
3HA:nup124ΔFXFG3-7*,FXFG1-2,8-11→ AAAA	Nup124 ^{ΔAA828-967+ AA571-574,612-615, 998-1001,1023-026,1089-1092, 1108-1111-AAAA}
3HA:nup124ΔFXFG	Nup124 ^{ΔAA571-1146}
Nup124FXFG	Nup124 ^{AA571-1146}
Nup153FXFG	Nup153 ^{AA903-1418}
Nup1FXFG	Nup1p ^{AA406-988}
Nup120p noFXFG	Nup120p ^{AA517-1137}
Nup124pΔFXFG:Nup153FXFG	Nup124p ^{ΔAA571-1111} -Nup153 ^{AA903-1418}
Nup124pΔFXFG:Nup1pFXFG	Nup124p ^{ΔAA571-1111} -Nup1p ^{AA406-888}
Nup124pΔFXFG:Nup120p noFXFG	Nup124p ^{ΔAA571-1111} -Nup120p ^{AA517-1137}
3HA:Nup124pR-A	3HA:nup124p ^{R1148A}
3HA:Nup124pRR-AA	3HA:nup124p ^{RR1156-57AA}
3HA:Nup124pΔRR	3HA:nup124p ^{Δ1156-57}
3HA:Nup124pΔCT	3HA:nup124p ^{Δ1147-59}
3HA:Nup124pΔCT*	3HA:nup124p ^{Δ1151-55}
Nup124pΔCT:Nup1pCT	Nup124p ^{ΔAA1147-59} :Nup1p ^{AA1065-76}
Nup124pΔCT:Nup153CT	Nup124p ^{ΔAA1147-59} :Nup153p ^{AA1464-1475}
Nup124pΔCT:Nup1pCT ⁺	Nup124p ^{ΔAA1147-59} :Nup1p ^{AA1065-1076+^R}
Nup124pΔCT:Nup153CT [®]	Nup124p ^{ΔAA1147-59} :Nup153p ^{AA1464-1475+^R}
Nup124pCT	Nup124p ^{AA1147-1159}
Nup1p+R	Nup1p ^{AA1-1076+^R}
Nup153ΔCT:Nup124CT	Nup153p ^{ΔAA1464-1475} :Nup124p ^{AA1147-59}
Nup124pCT*	Nup124p ^{AA1153RSRRKR1159-ASAAAA}
RFP:Nup120p seq	RFP:Nup120p ^{AA517-1137}
RFP:Nup124pFXFG	RFP:Nup124p ^{AA571-1111}
Nup124ΔCT*:Nup153CT [#]	Nup124p ^{ΔAA1151-55} :Nup153p ^{AA1468-72}
Nup124p ^m	Nup124 ^{AA1153RSRRKR1159-ASAAAA}
Nup124p [^]	3HA:nup124 ^{ΔAA571-1111::AA1151-1159}

on transposition activity. Thus, we conclude that the FXFG repeats themselves are not required for Tf1 activity.

FXFG-Repeat Domains of Nup1p and Nup153 Are Functional in Nup124p

In a previous report, we had identified the *S. cerevisiae* and vertebrate (human) nucleoporins Nup1p and Nup153, respectively, as orthologs of Nup124p (Varadarajan *et al.*, 2005). When Nup1p and Nup153 C-terminal regions are aligned independently with Nup124p C-terminal region as shown in Figure 2, there appears to be a low to moderate similarity/identity in regions besides the FXFG/FG repeats themselves. Because the entire FXFG-repeat-containing domain of Nup124p was required for Tf1 activity (Figure 1, patches 17 and 18; Balasundaram *et al.*, 1999; Varadarajan *et al.*, 2005), we wanted to know if such a requirement was inherent within the FXFG repeat-containing domain but not with the repeats themselves. One interesting possibility we pursued was a set of key observations by Rexach and co-workers that the large FG-repeat regions of Nups behaved in situ as natively unfolded structures thus giving them special features that may be critical for NPC function (Denning *et al.*, 2002, 2003; Denning and Rexach, 2007). In Figure 3A, we show that the percentage of “disorder amino acids” in the C-terminal half of Nup124p is comparable to its orthologs Nup1p and Nup153 but significantly higher than Nup120p, a nucleoporin that does not contain any FXFG/FG repeats. We therefore asked if the FXFG domains from Nup1p and Nup153 would functionally replace the FXFG region of Nup124p. As a control, the region from Nup120p lacking

either FG or FXFG repeats replaced the FXFG region of Nup124p. All the above chimeric proteins were expressed in *S. pombe* along with the Tf1 reporter plasmid, pHL449-1, to determine the levels of Tf1 activity by in vivo complementation of the defect in a null mutant as previously described (Balasundaram *et al.*, 1999; Varadarajan *et al.*, 2005). As seen in Figure 3B, the chimeras Nup124pΔFXFG-Nup153FXFG and Nup124pΔFXFG-Nup1pFXFG (see Table 2 for details) were able to rescue Tf1 activity comparable to wild-type levels. On the other hand, the Nup124pΔFXFG-Nup120p no FXFG chimera did not rescue Tf1 transposition. From Figures 1 and 3, we infer that although the Nup124p FXFG repeats themselves are not required, the FXFG domains of Nup124p (*S. pombe*), Nup1p (*S. cerevisiae*), and Nup153 (mammals) contain a common element(s) required for Tf1 activity.

Analysis of a Highly Conserved C-terminal Polypeptide GRKIAVPRSRKR of Nup124p That Is Absolutely Essential for Tf1 Transposition

In our initial experiments, deletion of the last 48 amino acids of Nup124p resulted in the abolition of Tf1 activity (unpublished results). Closer inspection of the three orthologs Nup1p, Nup124p, and Nup153 revealed a highly conserved sequence motif at the C-terminal end (Figure 4A). We therefore asked if this sequence, AA1147-GRKIAVPRSRKR-AA1159 was critical for Nup124p function and if so, which residues contributed to its activity. Amino acids were either deleted or replaced with alanine as indicated in Figure 4. B–D. Constructs were expressed in the null mutant along

Figure 1. FXFG repeats of Nup124p are not required for Tf1 activity. Multicopy plasmids containing full-length and mutant versions of Nup124 whose transcription was under the control of its native promoter or the low strength, *nmt81* (no message in thiamine) promoter were transformed into a $\Delta nup124$ mutant strain YNB19, along with the Tf1 reporter plasmid pHL449-1. Genetic assays measured the ability of these mutant strains to reinstate levels of Tf1 cDNA recombination and transposition to that of epigenetically expressed WT levels. PROT^{ts} (YNB10) containing a Tf1-reporter plasmid with a protease frame shift that blocks the expression of (Tf1) protease, reverse transcriptase, and integrase, 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 frame shift mutation. IN^{ts} (YNB11) contains a Tf1-reporter plasmid with a frame shift in the integrase so Tf1 transposition is decreased but not Tf1 cDNA recombination and serves as a negative control for the assay. *S. pombe* strains containing a Tf1-neo plasmid (pHL449-1) were grown as patches as shown, on EMM –ura dropout agar plates in the presence of thiamine (Tf1 Off) or in the absence of thiamine (Tf1 On). The transcription of the *nmt1*-Tf1-*neo* gene is induced in the absence of thiamine (Tf1 On). After 4 d of incubation at 32°C, these plates were replica printed to medium containing 5-FOA (100 mg/ml) to eliminate the *URA3*-Tf1-*neo* plasmid. Finally, 5-FOA⁺ patches were printed to medium containing both 5-FOA + G418 and incubated at 32°C for 38 h to detect strains that became resistant to G418 as the result of insertions of the *neo*-marked Tf1 element into the genome. For cDNA recombination assays, strains with the *neoAI*-marked Tf1 plasmid 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 YES medium that contained 500 μ g of G418/ml. Recombination between cDNA and cellular transposon sequences was scored on the G418 plates after 38 h of growth at 32°C. The numbering of the patches in the figure corresponds to strains diagrammatically represented in the bottom panel. Each oval shape in the diagrammatic representation indicates one FXFG repeat. (1) Epigenetically expressed WT (3HA:nup124), YNB58; (2) Null mutant containing an empty vector (*nmt81*:empty vector), YNB893; (3) PROT^{ts}, YNB10; (4) IN^{ts}, YNB11; (5 and 6) Nup124ΔFXFG1-2, YNB59; (7 and 8) Nup124ΔFXFG3-7, YNB235; (9 and 10) Nup124ΔFXFG8-9, YNB236; (11 and 12) Nup124ΔFXFG10-11, YNB65; (13 and 14) Nup124ΔFXFG3-7*, YNB 1086; (15 and 16) Nup124ΔFXFG3-7*, FXFG1-2,8-11 AAAAA, YNB1140; and (17 and 18) Nup124ΔFXFG, YNB1052 (see Table 2 for details).

with a Tf1 reporter plasmid, pHL449-1 (Table 2 and Supplementary Table S1) to determine the levels of Tf1 activity by genetic assays described earlier (Figure 1) As depicted in Figure 4B, the most dramatic results were associated with the 3HA:nup124pR-A (YNB1020), 3HA:nup124pRR-AA (YNB1070), 3HA:nup124pΔRR (YNB1034), and 3HA:nup124pΔCT (YNB1050) mutants, where complete elimination of Tf1 activity was noted. All other single amino acid changes yielded WT Tf1 activity and 3HA:nup124p ΔCT* (YNB1032) resulted in a significant lowering, but not elimination of Tf1 activity (Figure 4C), suggesting that the arginine residues at positions 1148 and 1156-1157 may constitute a bipartite nuclear localization signal (NLS) separated by the spacer “AVPRS.”

Because both Nup1p and Nup153 C-termini contain the equivalents of the Nup124p-R1148 and RR1156-57 residues described above, we wanted to know if the 12 amino acid sequences NRKIARMRHSKR (from Nup1p) or GRKIKTAVR-RRK (from Nup153) could functionally replace the 13 amino acids, GRKIAVPRSRRKR in Nup124p. Therefore, Nup124pΔCT:Nup1pCT (YNB1058) and Nup124pΔCT:Nup153CT (YNB1054) chimeras were generated and tested for support of Tf1 activity. Figure 4D shows that both chimeras were unable to promote Tf1 activity in a nup124 null background. We reasoned that the inability to rescue Tf1 activity was because of the disparity in the required numbers of amino acids in this domain. Another reason could be that because they do not have an amino acid corresponding to R1159 of Nup124p, they lacked a consensus “R” quorum in a contextual sense. We therefore added an arginine to the above chimeras to form Nup124pΔCT:Nup1pCT⁺ (YNB1062) and Nup124pΔCT:Nup153CT⁺ (YNB1215) and tested transformants for their ability to rescue Tf1 activity (Figure 4D). Interestingly, both chimeras restored Tf1 activity to wild-type levels. Our results are indicative of a specialized requirement fulfilled only by the Nup124p sequence GRKIAVPRSRRKR (Nup124pCT). We also noted that Nup1p+R (YNB1098) or Nup153ΔCT:Nup124CT (YNB1217) did not rescue Tf1 activity in their “native” Nup1p and Nup153 contexts, respectively, indicating that there may be other factors required for Tf1 activity besides the C-terminal peptide that are unique to Nup124p (Varadarajan *et al.*, 2005).

The Putative Gag-binding Domain, the FXFG-Repeat Domain and the C-terminal Peptide of Nup124p Are Independently Required for Nuclear Import of Tf1-Gag and Retrotransposon Activity

In earlier studies, we had demonstrated that Nup124p-mediated nuclear import of Tf1-Gag was critical for Tf1 activity and that deleting the putative Gag-binding or the FXFG-repeat domains caused a loss of Tf1 transposition, although the mutant proteins were localized at the NPC (Bala-sundaram *et al.*, 1999; Varadarajan *et al.*, 2005). We wanted to know if abrogation of Tf1 activity in the absence of the above domains correlated with the inability of these mutants to import Tf1-Gag into the nucleus. Null mutant strains episodically expressing Tf1-Gag:YFP cotransformed with plasmids containing 3HA-epitope-tagged WT (3HA:nup124) or mutant *nup124* sequences were tested for Tf1 activity. Nup124p and Tf1-Gag:YFP were identified in situ by epifluorescence microscopy. As depicted in Figure 5, in the presence of wild-type Nup124p, Tf1-Gag was found colocalizing with the DAPI stain, indicating that Gag was imported into the nucleus. On the other hand, in the presence of Nup124p mutations that delete N-terminus (Nup124p^{Δ111-331}; Varadarajan *et al.*, 2005), the entire C-terminus (nup124-1; Bala-sundaram *et al.*, 1999), the FXFG-repeat-containing domain (nup124ΔFXFG) or mutations in the C-terminal peptide, GRKIAVPRSRRKR (nup124ptm), Tf1-Gag was found local-

Figure 2. Sequence alignment of Nup124p, Nup1p, and Nup153 FXFG-repeat domains. The alignment of the C-terminal region of SpNup124p with the C-terminal domain of ScNup1p and HuNup153. Identical residues are boxed in black and similar/conserved residues in gray. Amino acid ranges are indicated at the start and finish of each sequence.

ized as cytoplasmic or perinuclear fluorescent aggregates. Similar cytoplasmic and perinuclear aggregation of Tf1-Gag has been observed (Balasundaram *et al.*, 1999; Varadarajan *et al.*, 2005) and may be a feature of VLPs (Teyssset *et al.*, 2003; Kim *et al.*, 2005). Tf1-gag localizes to the NPC, even in the absence of Nup124p (Varadarajan *et al.*, 2005). It is possible, therefore, that in the presence of Nup124p mutants, Tf1-Gag would localize at or close to the NPC, although it might not interact with it. Correspondingly, those mutants do not support Tf1 activity (Figure 5). We noted that none of the mutants exhibited any altered nuclear targeting or localization patterns when compared with the WT. Our results therefore suggest that all the above tested domains are critically important for importing Tf1-Gag into the nucleus as also to reconfirm our earlier conclusions that the Nup124-mediated translocation of Tf1-Gag from its perinuclear po-

sition into the nucleus was an absolute requirement for Tf1 retrotransposon activity.

Epigenetic Overexpression of Nup124p, Nup1p, and Nup153 FXFG/FG Domains or the Nup124p GRKIAVPRSRRRR Peptide in a Wild-type Strain Knockdown Tf1 Activity

A second approach to identifying the specificity of a domain required for activity was to observe if overexpression of FXFG-repeat-containing regions of Nup124p, Nup153, Nup1p, and the no-FXFG/FG-repeat region of Nup120p would cause an inhibition of Tf1 transposition in a WT background without inhibiting growth of the host. These experiments were performed based on the expectation that peptides whose overexpression interferes with Tf1-Gag function but do not influence

Figure 3. FXFG/FG-domains of Nup1p and Nup153 are functional in Nup124p in the context of Tf1 activity. (A) Disorder amino acids (% by frequency) in the indicated FXFG/FG region of SpNup124p, ScNup1p, HuNup153, and SpNup120p (a random non-repeat region corresponding to the C-terminal half of the protein) (B) Yeast strains expressing full-length Nup124p (YNB 1064), Nup124p with FXFG deletion (YNB 1052), chimeras of Nup124p with the indicated FXFG repeat (YNB 1117, Nup124pΔFXFG:Nup1pFXFG, or YNB 1096, Nup124pΔFXFG:Nup153FXFG), or a non-repeat-bearing chimeric fusion protein (YNB 1201, Nup124pΔFXFG:Nup120p no-FXFG) were assayed for Tf1 activity as described earlier. +++, Tf1 activity equivalent to the epigenetically expressed WT nup124 (YNB 1064); -, lack of Tf1 activity comparable to the null mutant (see Table 2 for details).

cell growth will act by disengaging Tf1-Gag from Nup124p. Such a dominant negative phenotype would be consistent with titration of an FXFG-domain-interacting protein by an excess of the Nup124p, Nup1p, or Nup153-FXFG-domain fragment. Also, it was anticipated that excess of Nup120p region with no FXFG/FG-repeats would not lead to a dominant negative inhibition of Tf1 activity because it did not support wild-type levels of Tf1 transposition as demonstrated in Figure 3B. Such a result would also imply a Tf1-related function rather than a global effect. Nucleotides encoding the FXFG regions of Nup124p, Nup1p, Nup153, and the no-FXFG region of Nup120 were cloned into a vector in which their transcription was under the control of the thiamine repressible *nmt1* promoter (highest strength; Table 2). These constructs were expressed in the WT along with a Tf1 reporter plasmid, pHL449-1 (Table 1) to determine levels of Tf1 activity. As depicted in Figure 6A, overexpression of FXFG/FG domains of Nup124p, Nup1p, and Nup153 (patches 5-6, 7-8, and 9-10, respectively) caused an equal and dramatic loss of Tf1 activity. Furthermore, no difference was observed in growth between Tf1-induced (No B1) or uninduced (+B1) cultures. In complete contrast, however, overexpression of the no-FXFG/FG region of Nup120p resulted in no loss of Tf1 activity (patches 11 and 12). Our results are consistent with observations that the FXFG domains of Nup1p and Nup153 act in a manner similar to the Nup124p FXFG domain, with respect to dominant negative inhibition of Tf1 activity. Our observation that a non-FXFG/FG- (Nup120p) nucleoporin chimera is unable to support wild-type levels of Tf1 transposition in a nup124 null mutant or knockdown transposition in a wild type when overexpressed, suggests a specific requirement of the FXFG domain.

We used the test described above to observe if overexpression of the Nup124p C-terminal peptide, GRKIA-VPSSRRKR (Nup124pCT) would cause a similar dominant negative inhibition of Tf1 transposition in a WT background. Nucleotides encoding the WT peptide sequence, GRKIA-VPSSRRKR and a random sequence, YAHSDATMVCMF5 were cloned into a vector in which their transcription was under the control of the thiamine repressible *nmt1* promoter (highest strength). The *nmt1*:RFP-GRKIAVPSSRRKR and *nmt1*:RFP-YAHSDATMVCMF5 constructs were expressed in the WT along with a Tf1 reporter plasmid, pHL449-1 (Table 1) to determine the levels of Tf1 activity by genetic assays described earlier. As depicted in Figure 6B, overexpression of *nmt1*:RFP-GRKIAVPSSRRKR caused a comprehensive knock down of Tf1 activity when compared with expression of the random sequence *nmt1*:RFP-YAHSDATMVCMF5. No difference was observed in growth between Tf1-induced (No B1) or noninduced (+B1) cultures. Taken together, our data demonstrate the Nup124pCT, GRKIA-VPSSRRKR sequence is specific and critical for Nup124p function in Tf1 retrotransposon activity.

Overexpression of the Nup124p FXFG/FG Domain or the Nup124p GRKIAVPSSRRKR in WT Blocks the Nuclear Import of Tf1-Gag

We wanted to investigate the biological basis for the knock-down of Tf1 activity described above. Because overexpression of these domains did not affect the general housekeeping attributes of the NPC to the extent at which growth would be adversely affected (Figure 6), it was safe to presume that the Nup124p FXFG/FG-repeat domain (Nup124p FXFG)

A				
NUP	Species	C-terminal peptide		
Nup124p	Sp	1147-GRKIAVPRSRKR-1159		
Nup1p	Sc	1065-NRKIARMRHSKR-1076		
Nup153	Hu	1464-GRKIKTAVRRRK-1475		
Nup153	Mus	1451-GRKIKTAVRRRK-1462		
Nup153	Rat	1588-GRKIKTAVRRRK-1599		
Nup153	Xe	1594-STRKIKTARRRK-1605		
Nup153	Gg	1461-VRKIKTAVRRRK-1473		

B		C		D	
C-terminal peptide	Tf1 Activity	NUP	+C-terminal peptide	Tf1 Activity	
1147					
↓					
GRKIAVPRSRKR	+++	Nup124p	GRKIAVPRSRKR	+++	
SRKIAVPRSRKR	+++	Nup124p	GRKIKTAVRRKR	+++	
GAKIAVPRSRKR	-	Nup124p	GRKIAVPRSRK	+++	
GRAIAVPRSRKR	+++	Nup124p	GRKI_____RRKR	+	
GRKAAVPRSRKR	+++				
_____AVPRSRKR	-				
GRKIAVPRSRKA	+++				
GRKIAVPRSRAR	+++				
GRKIAVPRSAKR	+++				
GRKIAVPRSARKR	+++				
GRKIAVPSARRKR	+++				
GRKIAVPRSRRAA	+++				
GRKIAVPRSRAAA	-				
GRKIAVPRSAAAA	-				
GRKIAVPRPAAAAA	-				
GRKIAVPAAAAAA	-				
GRKIAVPRSAAKR	-				
GRKIAVPRS_____KR	-				
-----	-				

Figure 4. Identification of a highly conserved peptide sequence xRKIxxxSRRKx at the C-terminus of SpNup124p, ScNup1p, and Nup153. (A) Sequence alignment of the C-terminal 13-amino acid region of Nup124p (AA 1147-1159), Nup1p, and Nup153 from various species. Representatives were chosen from a broad selection of eukaryotes: *S. pombe* (Sp), *S. cerevisiae* (Sc), Human (Hu), Mouse, *Mus musculus* (Mus), *Rattus norvegicus* (Rat), *Xenopus laevis* (Xe), and *Gallus gallus* (Gg). (B) Tf1 activity of wild-type and mutant Nup124p at the C-terminal polypeptide, GRKIAVPRSRKR (Nup124pCT) was measured as described in *Materials and Methods*. (C) The amino acids 1151-1155 (AVPRS) of the C-terminal polypeptide were mutated or deleted. Their effect on Tf1 activity was measured. (D) Tf1 activity was measured upon swapping the C-terminal peptide of Nup1p and Nup153 with the C-terminal peptide of Nup124p (YNB1058 and YNB1054, respectively), and swapping followed by addition of an arginine residue at the C-terminus (YNB1062 and YNB1215). Tf1 activity was also assayed in the presence of Nup1p (YNB 1017), upon addition of an arginine to Nup1p (YNB1098), Nup153 (YNB967) and upon swapping the C-terminal 12 amino acids of Nup153 with the C-terminal 13 amino acids of Nup124 (YNB1217; see Table 2 for details).

and the GRKIAVPRSRKR-peptide (Nup124pCT) were involved in a Tf1-specific function. Furthermore, because both domains were required for nuclear import of Tf1-Gag (Figure 5), it was possible that their overexpression would have a similar result because the domains would compete for the same sites as the native (wild-type) protein and as a consequence, Tf1-Gag would fail to be imported into the nucleus, resulting in loss of Tf1 activity. We therefore tested Tf1-Gag localization in wild-type strains overexpressing the Nup124p FXFG/FG-repeat domain (RFP:Nup124pFXFG) or the Nup120 sequence (RFP:Nup120pseq) in the presence of *nmt1*Tf1-Gag:YFP. Similarly, the Nup124CT (GRKIAVPRSRKR) or a random sequence, YAHSDATMVC MFS, were expressed as RFP-tagged peptides alongside YFP-tagged Tf1-Gag. The Nup120p sequence and the random sequence served as appropriate negative controls because their overexpression did not result in loss of Tf1 activity (see Figure 6). Strains harboring the above plasmids were grown in growth medium lacking thiamine to induce expression of Tf1 and processed for confocal fluorescence microscopy. Localization of Tf1-Gag:YFP is depicted in Figures 7 and 8. Overexpression of RFP:Nup120p seq does not affect the nuclear import of Tf1-Gag:YFP (Figure 7, row B). In fact, both RFP:Nup120p seq and Tf1-Gag:YFP colocalize in the nucleus (Nup120p seq has no canonical or characterized NLS,

and we noted that RFP:Nup120p seq remained outside the nucleus in 30% of fluorescing cells). In stark contrast, however, overexpression of RFP:Nup124pFXFG causes Tf1-Gag:YFP to be mislocalized outside the nucleus (Figure 7, row D). Furthermore, in all cells exhibiting both YFP and RFP fluorescence we observed a colocalization of the RFP:Nup124pFXFG with the Tf1-Gag:YFP outside the nucleus suggesting a close association of the Nup124p FXFG domain and Tf1-Gag. The colocalization of Nup124p FXFG domain with Tf1-Gag is consistent with our observation that the C-terminus of Nup124p does indeed bind Tf1-Gag (see below).

As described in this and earlier reports (Balasundaram et al., 1999; Varadarajan et al., 2005), Tf1-Gag:YFP localizes to the nucleus as shown in Figure 8, column A. The small peptides including the random sequence YAHSDATMVC MFS (column B) or the Nup124 C-terminal peptide GRKIAVPRSRKR (Nup124pCT; column C) shows a diffused localization pattern throughout the cell in the absence of Tf1-Gag:YFP, which is consistent with the diffusion of a small peptide fused to RFP. When RFP:YAHSDATMVC MFS and Tf1-Gag:YFP were expressed together, the latter was found in the nucleus (column D), consistent with the observation that overexpression of YAHSDATMVC MFS does not impede the nuclear import of Tf1-Gag and as a consequence

Figure 5. Mutations in Nup124p prevent Tf1-Gag from being imported into the nucleus resulting in loss of Tf1-transposon activity. YNB19 was transformed with Tf1-Gag:YFP and 3HA-tagged Nup124p (YNB549). Mutant Nup124p strains are shown, and their details provided in Table 2. Cells were grown in –Ura-Leu medium with or without 15 μ M thiamine. Cells were processed and visualized as indicated in *Materials and Methods*. Only cells grown in the absence of thiamine are shown. Strains shown were also tested for their ability to support Tf1 activity. +++, Tf1 activity equivalent to the epigenetically expressed WT (YNB549); –, lack of activity.

does not diminish Tf1 activity as shown in Figure 6. On the other hand, overexpression of RFP:GRKIAVPRSRKR together with Tf1-Gag:YFP (column E) results in blocking the nuclear import of Tf1-Gag:YFP, and results in its localization outside the nucleus. Absence of Tf1-Gag from the nucleus leads to loss of Tf1 activity (Figure 6). Taking our results from Figures 6–8 together, we demonstrate that epigenetic overexpression of Nup124p domains in a wild-type strain inhibit retrotransposon activity by effectively uncoupling Tf1-Gag from its nuclear import process.

Nup124p Interacts In Vitro with Tf1-Gag, the Importin β Kap95 and the Importin α 's Imp1p and Cut15

FG-Nups interact directly with a broad range of transport factors and there is compelling in vivo evidence that interaction between the carriers and FG-Nups is important for at least one stage of translocation through NPCs (reviewed in Ryan and Wente, 2000; Allen *et al.*, 2001; Stewart *et al.*, 2001). Studies have demonstrated interactions between FXFG or GLFG cores of nucleoporins and transport factors (Bayliss *et al.*, 2000, 2002a,b) and also binding of transport factors to non-FG regions of nucleoporins (Pyhtila and Rexach, 2003). Importin α , Kap60 (Srp1p) and importin β , Kap95 bind to the VXXRKIXXXRR-C-terminal motif of Nup1p (Floer *et al.*, 1997; Gilchrist and Rexach, 2003; Pyhtila and Rexach, 2003; Liu and Stewart, 2005), and the closely similar Nup153 C-terminal motif binds to importin α (Moroiu *et al.*, 1997). We were interested in understanding the possible involvement of transport factors in Nup124p mediated transport of Tf1-Gag across the nuclear membrane. Like its *S. cerevisiae* homolog, the *S. pombe* Kap95 (SPAC1B1.03c) is an essential

gene that encodes a protein localizing to the nuclear rim (Chen *et al.*, 2004). In contrast to the budding yeast's single importin α , fission yeast has two importin α s, Cut15p and Imp1p, which have partially overlapping and distinct functions (Matsusaka *et al.*, 1998; Umeda *et al.*, 2005). We wanted to know if Nup124p's role in nuclear import of Tf1 (Gag), vis-à-vis Tf1 transposition, was contingent on its interaction with Kap95 and/or one or both importin α . We performed in vitro binding assays and coimmunoprecipitation with Nup124p, Tf1-Gag, Kap95, Cut15p, Imp1p, Grn1p, and HIV-1 Gag proteins synthesized using a TNT-coupled reticulocyte lysate system. In Figure 9A, we show that full-length Tf1-Gag (lanes 1 and 2), Imp1p (lanes 3 and 4), Cut15p (lanes 5 and 6), and Kap95 (lanes 7 and 8) bind independently to full-length Nup124p. To establish that the above interactions were bonafide and specific, we showed that Nup124p did not coimmunoprecipitate with either p53 or SV40 (lanes 9 and 10). Similarly, a nucleolar protein Grn1p (Du *et al.*, 2006) does not interact with either Tf1-Gag or Nup124p (Figure 9C, lanes 1-2 and 3-4, respectively). In Figure 9B we show that Tf1-Gag binds both, the N- and C-terminal halves of Nup124p. Although the N-terminus binding to Tf1-Gag reconfirms our earlier conclusions (Balasundaram *et al.*, 1999; Varadarajan *et al.*, 2005) and those of Kim *et al.* (2005), the C-terminal half of Nup124p (containing the entire FXFG/FG repeat domain) binding to Tf1-Gag reverses our earlier conclusion that no binding was observed for Nup124p^{AA 522-1152} to Tf1-Gag (Balasundaram *et al.*, 1999). It is possible that the binding of the C-terminal (AA522-1152) of Nup124p to Tf1-Gag could be indirect, mediated via importins, an integral part

Figure 6. Overexpression of the FXFG domain (Nup124pFXFG) or GRKIAVPRSRKR (Nup124pCT) peptide sequence of Nup124p inhibits Tf1 activity without significantly affecting growth. (A) Genetic assays were conducted exactly as described in Figure 1 so as to measure the ability of epigenetically expressed mutants (patches 5-12) to reinstate levels of Tf1 activity in YNB16 (wild type). The indicated strains were grown in EMM –Ura-Leu+B1 (Tf1-off) and EMM –Ura-Leu-B1 (Tf1-on), and growth was measured as a function of optical density (OD 600 nm). Details of the indicated constructs provided in Table 2. (B) Multicopy plasmids containing fragments encoding the indicated amino acids, GRKIAVPRSRKR from Nup124p (Nup124pCT) and a random sequence, YAHSDATMVCMF5, whose transcription was under the control of the high strength, *nmt1* promoter were tested in a WT strain (YNB16) for their effect on Tf1 activity. (1) Null mutant containing an empty vector served as a negative control, (YNB893); (2-5) *nmt1*:GFP, YNB1253; (6-9) *nmt1*:GFP-GRKIAVPRSRKR, YNB1066; (10-17) *nmt1*:GFP-YAHSDATMVCMF5, YNB1219; (18) PROT^{ts}(YNB10) described earlier (19) IN^{ts}(YNB11) described earlier. The indicated strains were grown in EMM –Ura-Leu+B1 (Tf1-off) and EMM –Ura-Leu-B1 (Tf1-on), and growth was measured as a function of optical density (OD 600 nm; see Table 2 for details).

of reticulocyte lysates. The known interaction between SV40 and p53 was performed routinely as a positive control to monitor the efficacy and robustness of the *in vitro* interaction assay (Figure 9B, lane 5).

DISCUSSION

The C-terminal Halves of Nup124p (S. pombe), Nup1p (S. cerevisiae), and Nup153 (Human) Contain Common Elements That Are Essential for Tf1 Activity

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 (Varadarajan *et al.*, 2005). Poor sequence conservation between FG Nups throughout eukarya, is suggestive of rapid evolution (Denning and Rexach, 2007).

Nup124p shares certain sequence and functional features with Nup1p and Nup153 (Cronshaw *et al.*, 2002; Hase and Cordes, 2003; Ball and Ullman, 2005; Varadarajan *et al.*, 2005). One of those features, the FXFG-repeat domain spans a major portion of the C-terminus (see Figure 6A in Varadarajan *et al.*, 2005). Although full-length Nup153 does not complement Nup124p, a region of Nup153 (aa448-634) was found capable of substituting for a domain within Nup124p (Varadarajan *et al.*, 2005). Nucleoporins containing the FXFG variant of the FG motif have more disorder amino acids than Nups containing mostly GLFG or xxFG motifs (Denning *et al.*, 2003). FXFG-repeat-containing nucleoporins, such as Nup1p, Nup2p, and Nup60p are located in the nuclear basket structure (Rout *et al.*, 2000) and mostly bind Kap95p-Kap60p heterodimers (Allen *et al.*, 2001). In vertebrates, it has been shown that either the FXFG nucleoporins or proteins bound to them are required for transport (Finlay and Forbes, 1990).

Figure 7. Tf1-Gag is mislocalized upon over expression of the Nup124p, Nup1p, and Nup153-FXFG/FG domains in the WT strain. YNB16 was transformed with RFP:Nup124pFXFG (YNB1223) and RFP:Nup120p seq (YNB1221) along with *nmt1*:Tf1-Gag:YFP. YNB 16 was also transformed with RFP:Nup124pFXFG (YNB1232) and RFP:Nup120p seq (YNB1230) alone (see Table 2 for details). Cells were grown in EMM –Ura-Leu medium with or without 15 mM thiamine. Only cells grown in the absence of thiamine are shown.

Our initial premise for assuming a Tf1-specific role for the FXFG-repeat domain was threefold. First, Nup124p is one of several FXFG-containing nucleoporins in fission yeast and yet its specific requirement was crucial in supporting Tf1 activity. Second, the FXFG domain was required for Tf1 activity (Varadarajan *et al.*, 2005) but not for recruitment of Tf1-Gag to the nucleus (Balasundaram *et al.*, 1999; Varadarajan *et al.*, 2005). Finally, in the absence of the FXFG domain, Tf1-Gag failed to be translocated into the nucleus (Figure 5). Deleting sections of the FXFG-repeat domain that included FXFGs 1-2, 3-7, 8-9, or 10-11 did not affect Tf1 activity. We assumed that the repeats were redundant functionally and thus compensated each other. We therefore used the largest deletion construct 3HA:nup124ΔFXFG3-7* to substitute the remaining FXFGs 1, 2, 8, 9, 10, and 11 with alanine tetrapeptides. This strain which has no FXFG repeats, had no effect on Tf1 activity either, implying that the FXFG repeats themselves had an insignificant role in Nup124p-mediated nuclear import of Tf1. If not the FXFG repeats themselves, it could be some other motif inherent in stretches between the FXFG repeats or a signature constituting the entire domain that is required for function.

In vivo studies implicate non-FG-Nups in control of permeability across the nuclear membrane (Shulga and Goldfarb, 2003). Furthermore, Kap95-binding sites in the non-FG domains of Nup60 (Denning *et al.*, 2001) and Nup1p (Pyhtila and Rexach, 2003) have been documented. The

asymmetric FG repeats of Nup1p and Nup159 are not required for Kap95-mediated import, Xpo1-mediated export, or mRNA export (Zeitler and Weis, 2004). Similarly, export of heat-shock RNAs are mediated by the unique carboxy-terminus of Rip1p, with no substantial contribution from the FG-repeat region (Stutz *et al.*, 1997). These findings raise the interesting possibility that Tf1-Gag import is mediated through transport molecules binding to high-affinity non-FG-binding sites found within the C-terminus of Nup124p.

Various models have been proposed in order to explain translocation across the nuclear membrane. An interacting meshwork of FG repeats forms a hydrophobic sieve blocking translocation of molecules not bound by transport factors (Ribbeck and Gorlich, 2001, 2002). Transport factors (specifically, importin β) bind sequentially to different FG domains with progressively increasing affinity from one NPC side to the other, with highest affinity sites at respective exit points (Ben-Efraim and Gerace, 2001; Pyhtila and Rexach, 2003). An elaborate study in *S. cerevisiae* demonstrated redundancy in the FG domains, that a large proportion of the FG domain mass could be eliminated without affecting NPC permeability. Indeed, in that same study, it was shown that the entire FG content of Nup1p (localizing to the nuclear side of NPC) could be eliminated without loss of nuclear import function or viability (Strawn *et al.*, 2004). Of the eight FXFG/FG-repeat Nups in *S. pombe* (Denning and Rexach, 2007), three including Nup124p are not essential for cell

Figure 8. Tf1-Gag is mislocalized upon over expression of the C-terminal GRKIAVPRSRKR (Nup124pCT) polypeptide in a WT strain. YNB16 was transformed with *nmt1*:RFP-GRKIAVPRSRKR (YNB1198) or *nmt1*:RFP-YAHSDATMVCMF5 (YNB1191) along with *nmt1*:Tf1-Gag:YFP. YNB16 was transformed with *nmt1*:RFP-GRKIAVPRSRKR (YNB1197), *nmt1*:RFP-YAHSDATMVCMF5 (YNB1189) and *nmt1*:Tf1-Gag:YFP (YNB1234) alone. Cells were grown in EMM –Ura-Leu medium with or without 15 mM thiamine. Only cells that were induced are shown (see Table 2 for details).

viability (Balasundaram *et al.*, 1999; Chen *et al.*, 2004). Of all the nonessential nucleoporins tested, only Nup124p was required for Tf1 transposition (unpublished data). Therefore, the requirement for the FXFG/FG domain of Nup124p by Tf1 and its functional replacement only by the Nup1p or Nup153 domains (and not by the FG/FXFG-less Nup120p), is intriguing and indicative of conservation of function. Although these results might suggest a specific nature of Nup124p's FXFG-domain requirement, it is possible that Nup124p interacts in a similar manner with proteins, such as transport factors, other nucleoporins, none of which are essential for viability and therefore unaffected by overexpression of the FXFG domain. The growth rate is not affected upon overexpressing these domains, indicating that essential functions were not compromised, even if overexpression were to dislodge cargoes from other FXFG-containing nucleoporins. Special features on nucleoporins (like Nup124p) may be needed to import large macromolecular cargoes like the virus-like particles (VLP) of Tf1 (Kim *et al.*, 2005). Such features may be closely related to the observation(s) that Nup1p and Nup153 contain a significantly higher number of "disorder-promoting" amino acid residues, are natively unfolded, display an extended conformation, are flexible, practically lack a significant secondary structure, or are devoid of any possible steric hindrances (Dunker *et al.*, 2001; Fahrenkrog *et al.*, 2002; Denning *et al.*, 2003; Paulillo *et al.*, 2005; Denning and Rexach, 2007; Lim *et al.*, 2006). Such qualities are likely to confer the nucleoporin with

multiple binding capacities that allow them to act as large surface-area scaffolds for assemblages of nuclear pore complexes (Paulillo *et al.*, 2005; Denning and Rexach, 2007) or hydrogel-like elastic properties that would enhance fluid movement within the NPC (Frey *et al.*, 2006). Like its orthologs Nup153 and Nup1p, the fission yeast Nup124p has a significantly higher frequency (%) of disorder-promoting residues at its C-terminus (FXFG-domain end; Figure 3A; see also Supplementary Table S3 of Denning and Rexach, 2007) when compared with Nup120p, which possibly explains why the respective domains from Nup153 and Nup1p may functionally replace the FXFG-repeat region of Nup124p, whereas the Nup120p sequence does not. In addition to earlier reports that the N-terminal region of Nup124p is a critical determinant of its association with Tf1-Gag (Balasundaram *et al.*, 1999; Kim *et al.*, 2005), in vitro-binding assays reported in this study suggest that there may be more than one point-of-contact between the two proteins because both the N- and C-termini of Nup124p bind Tf1-Gag (Figure 9). It is possible that while the interaction between Tf1-Gag and the N-terminus of Nup124p is direct, the interaction with the C-terminus is indirect, mediated via other factors, such as importin α - β recognition of Gag NLSs.

The extended FXFG-rich C-terminal half of Nup153 is highly mobile (Fahrenkrog *et al.*, 2002) and might increase the efficiency of cargo translocation thorough the NPC (Stoffler *et al.*, 2006). Given the ability of the 50-nm Tf1 particles to traverse the pore (Teyssset *et al.*, 2003), one could

Figure 9. Coimmunoprecipitation of Nup124p with Tf1-Gag, Kap95, Imp1p, and Cut15p from rabbit reticulocyte lysates. (A) The following sequences: Nup124, Tf1-Gag, Kap95, Imp1, Cut15, and Grn1:GFP from BNB401, BNB706, BNB659, BNB704, BNB702, and BNB373, respectively (Supplementary Table S1), were transcribed and translated in rabbit reticulocyte lysates in the presence of [35 S]methionine. Translated products were mixed in various combinations as indicated. Coimmunoprecipitation assays were performed as described in *Materials and Methods* with the appropriate antibodies, as indicated. (B) Nup124AA1-570 (BNB566) and Nup124 AA571-1159 (BNB761) were transcribed and translated using the rabbit reticulocyte lysate in the presence of [35 S]methionine. The products were incubated with Tf1-Gag (BNB706) and immunoprecipitated with antibodies as indicated. (C) Grn1p (BNB338), a nucleolar protein of *S. pombe* was incubated with Nup124 (BNB401) and Tf1-Gag (BNB706) and immunoprecipitated with antibodies as indicated in *Materials and Methods*. The immunoprecipitates were resolved on 4-15% SDS-PAGE and detected by fluorography. The relative mobilities of full-length/+tag proteins are indicated by arrows.

envisage the critical importance of an extended FXFG-repeat domain of Nup124p in engaging Tf1-Gag at the NPC in translocating the same into the nucleus. Because Nup124p binds to Tf1-Gag (Figure 9; Balasundaram *et al.*, 1999; Varadarajan *et al.*, 2005), it is possible that the Nup124p FXFG-repeat domain may be crucial in conjunction with its N-terminus in serving as a large surface-area receptor for the 50-nm Tf1 particle.

The Conserved Sequence Motif GRKIAVPRSRKR at the C Terminus of Nup124p (Nup124p^{AA1147-1159}) Is a Critical Determinant of Tf1 Activity

The Nup124p C-terminal sequence motif GRKIAVPRSRKR (Nup124pCT) closely resembles the C-terminus of Nup1p (VMXXRKIA), Nup153 (VXXRKIXXXRRK; Figure 4A), the KaRF (karyopherin release factors) motifs present at the N termini of Nup2p (VMXXRKIAXXKRR), and human Nup50

(VXXXRAIKXXRR; Gilchrist and Rexach, 2003). Mutation studies (Figure 4) clearly establish the singular importance of the arginine residue in the RKIA tetrapeptide because the 3HA:nup124pR-A mutant lacked the ability to support Tf1 activity. Similarly, the RR basic residues in the tetrapeptide RRRK (AA1156-1159) were critically important. All four basic residues were dispensable when deleted one at a time, suggesting functional redundancy. The sequence motif in general resembles a bipartite NLS with two basic peptide motifs separated by an interchangeable spacer sequence. Indeed, the motif does possess the ability to transport a GFP-LacZ fusion protein into the nucleus (unpublished data). When we replaced the intervening (spacer) sequence with the KTAVR from the Nup153 intervening sequence (Nup124 Δ CT*:Nup153CT $^{\#}$), no loss of activity was observed, but deleting the same resulted in a significant drop in Tf1 activity. Replacing the Nup124p motif GRKIAVPRSRKR (Nup124pCT) with either Nup1p or

Nup153 C-terminal sequence motifs did not generate WT levels of Tf1 activity. Addition of an arginine residue to the termini of the above Nup124p-chimeras restored WT levels of Tf1 activity that may represent a gain-of-function mutation in these chimeras, although ironically, this arginine residue itself was not essential in the WT Nup124p context. Indeed, previous studies (Booth *et al.*, 1999; Gilchrist and Rexach, 2003) demonstrated that a very similar sequence in *S. cerevisiae* Nup2 **RRKIAMPKRRMAFK** can actively displace NLSs from importin α , whereas the very similar, endogenous Nup1 sequence **NRKIARMRHSKR** cannot and instead, provides a docking site for importin α - β -cargo complexes. Because *S. pombe* does not appear to have a Nup2 (Chen *et al.*, 2004; Denning and Rexach, 2007), an intriguing possibility is that the C-terminus of Nup124 retains this critical Nup2 activity. Human Nup153 and *S. cerevisiae* Nup1 may have lost that activity during evolution, given their content of active Nup50 and Nup2p, respectively. The full-length Nup1p and Nup153 with an arginine added at the C-terminus did not rescue Tf1 activity in the Nup124 *null* mutant possibly because Nup1p lacks the putative Gag-binding domain and Nup153 has other components that could adversely affect Tf1 transposition (Varadarajan *et al.*, 2005).

The C termini of Nup1p (¹⁰⁶⁵NRKIARMRHSKR¹⁰⁷⁶) and Nup153 (¹⁴⁶⁴GRKIKTAVRRKK¹⁴⁷⁵) are known to directly bind Kap60 and importin α 2, respectively, and in the case of Nup1p, is a part of the high-affinity binding site for the importin β homolog, Kap95 (Moroiu *et al.*, 1997; Gilchrist and Rexach, 2003; Pyhtila and Rexach, 2003; Liu and Stewart, 2005). Our observation that adding back an arginine to the existing Nup1p-¹⁰⁶⁵NRKIARMRHSKR¹⁰⁷⁶ or Nup153 ¹⁴⁶⁴GRKIKTAVRRKK¹⁴⁷⁵ restores Tf1 activity suggests a conservation of function. Additionally, our *in vitro*-binding studies suggest that like its orthologs, Nup124p interacts with the fission yeast transport factors importin β , Kap95 and the two importin α s, Cut15p and Imp1p. However, because *Kap95* and *Cut15* are essential genes in *S. pombe* (Matsusaka *et al.*, 1998; Chen *et al.*, 2004; Umeda *et al.*, 2005), we could not test their role(s) in Tf1 transposition. On the other hand, deleting the nonessential Imp1p did not result in reduced Tf1 function (unpublished data). This is possibly because Cut15p could replace Imp1p functionally, because both importin α s are known to have overlapping functions (Umeda *et al.*, 2005).

Knockdown of Retrotransposon Activity by Disengaging Tf1-Gag from Its Host Nuclear Transport Machinery

Overexpression of either the FXFG-repeat domain or the C-terminal peptide of Nup124p comprehensively knocked down Tf1 activity in the presence of a WT copy of the gene. This dominant negative inhibition was not observed when the Nup120p or a random peptide sequence was overexpressed, suggesting the involvement of a highly specific mechanism-based inhibition. On the basis of our observation(s) that these domains of Nup124p may be required for the nuclear import of Tf1-Gag and that the nuclear uptake of the latter was a prerequisite for Tf1 activity, we assumed that overexpressing them would likely affect Tf1-Gag localization. Although sequences from Nup120p, a nucleoporin with no FXFG/FG-repeats or a random sequence, YAHS-DATMVMCMFS did not prevent Tf1-Gag from entering the nucleus, cells overexpressing either FXFG-repeat region of Nup124p, RFP:Nup124pFXFG, or the Nup124pCT, RFP:GRKIAVPRSRKR, showed that Tf1-Gag:YFP was not imported into the nucleus or at least not accumulated within the nucleus at concentrations that would enable Tf1 transposition to occur. Interestingly, in a majority (>90%) of these cells, Tf1-Gag:YFP is typically disengaged from the medially

placed interphase nucleus and appears closer to either or both polar ends. An intriguing possibility is that the FXFG-repeat domain does in fact, bind to some cytoplasmic component(s) because it localizes in large aggregates at distal locations from the nucleus even in the absence of Tf1-Gag (Figure 7C). We noted that such localization is not without some specificity, because overexpression of the Nup120-domain does not localize to those same areas. Moreover, under certain conditions, the cytoplasmic accumulation of nuclear proteins could alter functions in viral translation, RNA synthesis, packaging, or even assembly (Gustin and Sarnow, 2002). Because Tf1-Gag is recruited to the nuclear rim/perinuclear area in the absence of Nup124p (Varadarajan *et al.*, 2005) or in the presence of nup124 mutants (Figure 5), the large cytoplasmic aggregates that we observe when either one of the two domains is overexpressed may prevent Tf1-Gag from “hitching” its natural ride to the nucleus. Clearly, an understanding of this process will shed light on some of the major upstream events from the formation of the virus-like particle in the cytoplasm to its recruitment at the nucleus. Though Nup124p is at the NPC/nuclear membrane throughout the cell cycle and nuclear division (unpublished data), our current over expression data uncover a link between upstream elements far removed from the nucleus that may have a bearing on Tf1-Gag being imported into the nucleus. Lastly, as in the case of Nup124p, Nup1p, and Nup153 conserved domains, there are likely to be other similarly conserved host nuclear transport factors that may be manipulated to serve as biological (host-mediated) attenuators affecting either specific or large groups (families) of viruses that have similar modes of intracellular transport.

ACKNOWLEDGMENTS

We thank Padmapriya Varadarajan and Ng Su Ying for technical assistance. This study was funded by the Agency for Science, Technology, and Research (A*STAR), Republic of Singapore.

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