# The FG-repeat asymmetry of the nuclear pore complex is dispensable for bulk nucleocytoplasmic transport in vivo

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Nucleocytoplasmic transport occurs through gigantic proteinaceous channels called nuclear pore complexes (NPCs). Translocation through the NPC is exquisitely selective and is mediated by interactions between soluble transport carriers and insoluble NPC proteins that contain phenylalanine-glycine (FG) repeats. Although most FG nucleoporins (Nups) are organized symmetrically about the planar axis of the nuclear envelope, very few localize exclusively to one side of the NPC. We constructed *Saccharomyces cerevisiae* mutants with asymmetric FG repeats either deleted or swapped to generate NPCs with inverted FG asymmetry. The mutant Nups localize properly within the NPC and exhibit exchanged binding specificity for the export factor Xpo1. Surprisingly, we were unable to detect any defects in the Kap95, Kap121, Xpo1, or mRNA transport pathways in cells expressing the mutant FG Nups. These findings suggest that the biased distribution of FG repeats is not required for major nucleocytoplasmic trafficking events across the NPC.

## Introduction

The nuclear pore complex (NPC) spans the two lipid bilayers of the nuclear envelope and is the essential mediator of all known transport events between the nucleus and the cytoplasm. Whereas the overall architecture and structure of the NPC is well conserved between species, its estimated size varies from ~50 MD in yeast to ~125 MD in amphibia (Hinshaw et al., 1992; Akey and Radermacher, 1993; Yang et al., 1998). Despite its enormous mass, the NPC of both yeast and humans consists of only ~30 distinct proteins, termed nucleoporins (Nups; Rout et al., 2000; Cronshaw et al., 2002). The core of the NPC is highly symmetric about the central plane of the nuclear envelope, such that most Nups can be found on both the cytoplasmic and nuclear faces of the pore (Rout et al., 2000). However, the yeast NPC also contains at least five asymmetrically positioned Nups, and the vertebrate NPC contains highly asymmetric filaments and basket structures on the cytoplasmic and nuclear side, respectively (for reviews see Rout and Aitchison, 2001; Vasu and Forbes, 2001; Suntharalingam and Wente, 2003).

Approximately one third of all Nups contain a conserved sequence motif of phenylalanine-glycine (FG) repeats (Rout et al., 2000; Cronshaw et al., 2002). In general, these FG repeats are made up of 4–48 GLFG, FxFG, SxFG, or PxFG motifs that are separated by spacers of variable length. FG Nups appear to play a central role in mediating the translocation of transport receptor–cargo complexes through the NPC by providing important interaction surfaces for transport factors. All known classes of transport receptors specifically interact with FG Nups, including members of the karyopherin/importin β superfamily, the mRNA export receptor Mex67/Mtr2, and the Ran transporter Ntf2 (for reviews see Lei and Silver, 2002; Weis, 2003). Indeed, the binding of transport factors to FG repeats is required for active transport through the NPC (Bayliss et al., 2000; Bednenko et al., 2003).

One of the biggest challenges remaining in the field is to understand how the NPC operates. Several models have been proposed to explain the vectoriality of nuclear transport and the selectivity of the NPC channel. To account for the permeability barrier of the NPC, the "selective phase model" was suggested (Ribbeck and Gorlich, 2001), which proposes weak interactions between FG Nups form a tight meshwork that excludes the transport of large macromolecules. Translocation occurs when the inter-FG Nup interactions are dissolved by transport factors that specifically interact with FG repeat domains (Ribbeck and Gorlich, 2001; Ribbeck and Gorlich, 2002). Alternatively, the "Brownian affinity gate" model proposes that docking to peripheral FG Nups facilitates entry into the

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Abbreviations used in this paper: cNLS, classical NLS; EB, extraction buffer; FG, phenylalanine-glycine; NES, nuclear export signal; NPC, nuclear pore complex; Nup, nucleoporin; RFP, red fluorescent protein.


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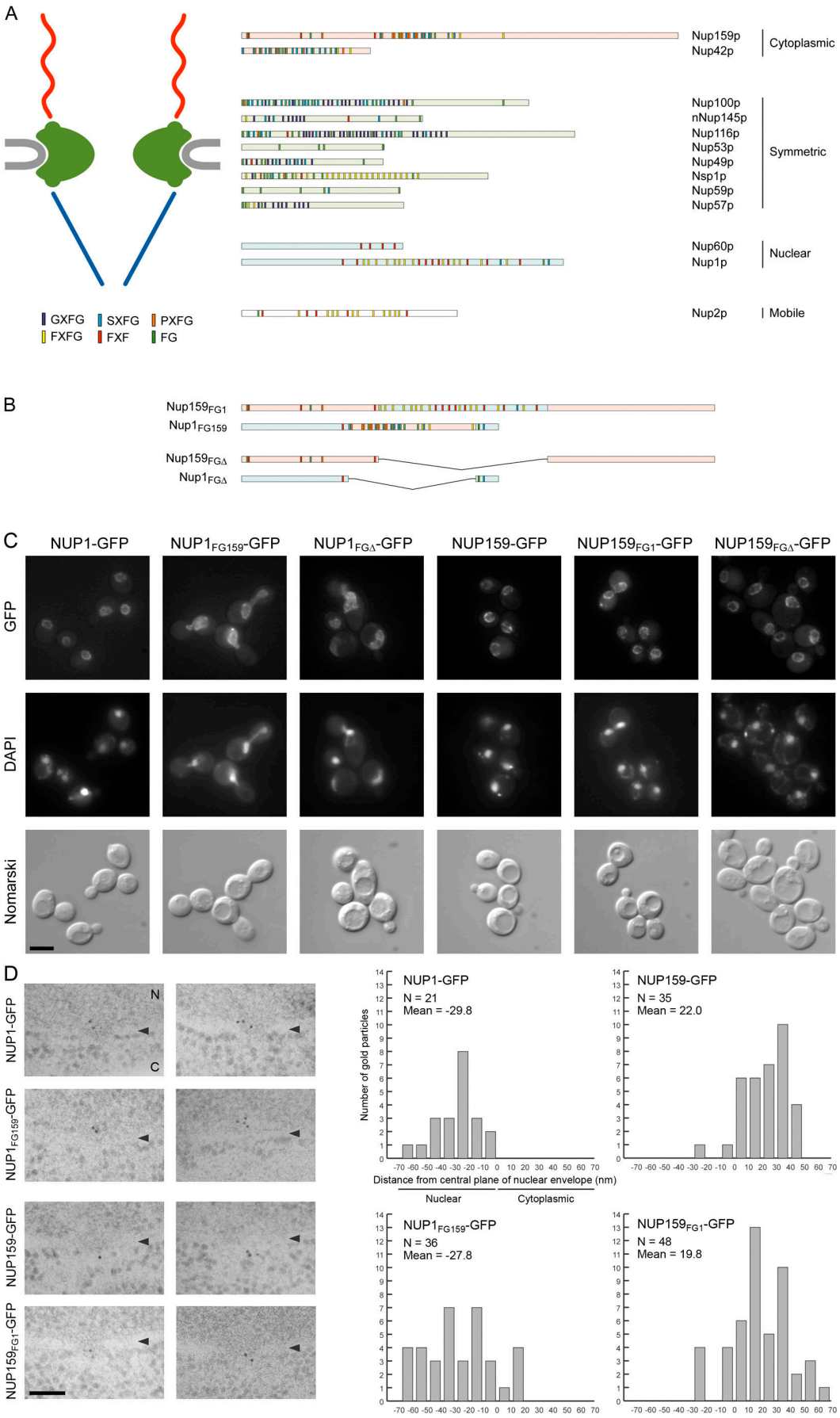
A
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NUP159-GFP
N = 35
Mean = 22.0
Number of gold particles
Distance from central plane of nuclear envelope (nm)
Nuclear
Cytoplasmic
NUP1FG159-GFP
N = 36
Mean = -27.8
NUP159FG1-GFP
N = 48
Mean = 19.8

narrow NPC channel and translocation occurs via random motion. Vectorial transport is achieved by a combination of the asymmetric arrangement of Nups and the asymmetric release of cargo (e.g., triggered by RanGTP; Rout et al., 2000). Consistent with this model is the finding that the highest affinity NPC binding sites for transport receptors are commonly found at the peripheral, asymmetric Nups (Allen et al., 2001, 2002; Ben-Efraim and Gerace, 2001; Pyhtila and Rexach, 2003). These observations led to the proposal that a gradient of increasing affinities between transport receptors and Nups along the NPC contributes to the directionality or efficiency of nuclear transport (Ben-Efraim and Gerace, 2001; Pyhtila and Rexach, 2003).

In an attempt to dissect the function of the NPC in vivo, we have generated FG-domain mutants and have swapped FG repeats between asymmetrically localized Nups in yeast. Examination of multiple transport pathways in these mutants suggests that asymmetrically positioned FG domains are dispensable for bulk transport across the pore.

## Results and discussion

### Mutant FG alleles localize properly within the NPC

As illustrated in Fig. 1 A, the composition of FG repeats varies and exhibits a biased distribution across the pore. To understand the functional importance of the asymmetric distribution of FG repeats, we generated yeast strains with the FG repeats of the cytoplasmic Nup159 and nuclear Nup1 deleted or swapped (Fig. 1 B). Nup159 and Nup1 preferentially bind to the transport factors Xpo1 (Allen et al., 2002; unpublished data) and Kap95 (Allen et al., 2001; Pyhtila and Rexach, 2003), respectively. We predicted that swapping FG repeats to the opposite side of the pore would create dominant NPC alleles with exchanged specificity for transport factors and thus would reverse the FG gradient.

We first confirmed the in vivo localization of the Nup1 and Nup159 FG mutants. GFP-tagged versions of the *NUP1* and *NUP159* alleles were expressed under the control of their endogenous promoters in *nup1*Δ or *nup159*Δ strains. Both the wild-type and mutant FG alleles displayed stereotypical nuclear envelope staining when visualized by fluorescence microscopy (Fig. 1 C), indicating that the FG-repeat regions of Nup1 and Nup159 are not required for proper localization to the NPC. To test whether the FG swap affected the asymmetric distribution of Nup1 or Nup159, we also determined their position within the NPC using indirect immuno-EM. As demonstrated previously (Kraemer et al., 1995; Rout et al., 2000), Nup159 localized exclusively to the cytoplasmic side of the NPC, whereas Nup1 is only detected at the nuclear surface (Fig. 1 D). Importantly, the exchange of the FG repeats between the two proteins did not affect their localization within the NPC, because $Nup159_{FG1}$ remained cytoplasmic and $Nup1_{FG159}$ was highly enriched on the nuclear side of the NPC (Fig. 1 D). Thus, we were able to delete multiple FG domains and specifically localize the FG repeats of Nup1 and Nup159 to the "wrong" side of the NPC without disturbing the overall organization of the pore.

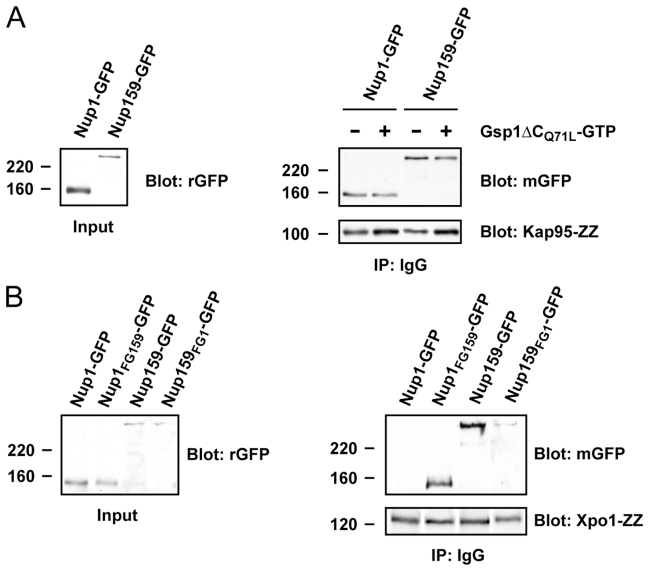



Figure 2. **In vivo association of Kap95 and Xpo1 with the mutant FG alleles.** (A) Whole cell extracts of strains expressing Kap95-ZZ were preincubated with 10 μM Gsp1ΔC$_{Q71L}$-GTP or a mock treatment, immunoprecipitated, and detected by Western blot. (Left) 4 μg of input from each Kap95-ZZ strain was loaded and blotted with a highly sensitive rabbit anti-GFP antibody (rGFP; Seedorf et al., 1999). (Right) One fifth of the total bead volume was loaded from each pull down (± Gsp1ΔC$_{Q71L}$-GTP) and blotted with highly specific mouse anti-GFP (mGFP; top) or anti-Kap95 antibodies (bottom). (B) Whole cell extracts of strains expressing Xpo1-ZZ were processed and detected as above for the Kap95-ZZ extracts. 10 μM Gsp1ΔC$_{Q71L}$-GTP was included in all pull-down reactions. (Left) 4 μg of input from each Xpo1-ZZ strain was loaded, blotted, and detected with rGFP. (Right) One fifth of the total bead volume was loaded from each pull down and blotted with mGFP (top) or anti-Xpo1 antibodies (bottom). Note that all lanes are derived from the same blot and exposure, but have been repositioned for clarity.

Figure 1. **Mutant FG alleles correctly localize within the NPC.** (A) The cartoon (left) depicts the overall architecture of the NPC embedded in the nuclear envelope. Shown are the cytoplasmic filaments (red), central channel (green), and nuclear basket (blue), as well as the corresponding FG Nups (right) from which these structures are partly derived (adapted from Rout et al., 2000). Note that PxFG/SxFG repeats are mainly found in cytoplasmic Nups, GxFG repeats are present in the Nups forming the central channel, and FxFG and FxF repeats are enriched in nuclear Nups. (B) The FxF/FxFG repeats of Nup1 (aa 401–967) were amplified by PCR and cloned into Nup159, replacing its FG repeat domain (aa 458–902, $Nup159_{FG1}$). Similarly, the SxFG/PxFG repeat domain of Nup159 (aa 459–885) was used to replace the Nup1 FG repeats (aa 358–1,000, $Nup1_{FG159}$). Additionally, alleles were created that completely lack the FG-repeat domains (deletion junctions are the same as in the FG-swap alleles). (C) Log-phase cultures were stained with DAPI and visualized by bright-field and fluorescence microscopy. Bar, 5 μm. (D) Log-phase cells expressing GFP-tagged FG alleles were prepared for immuno-EM by high pressure freezing (McDonald and Muller-Reichert, 2002). (Left) Representative images showing thin sections stained for the GFP epitope and labeled with a gold-conjugated secondary antibody. Nuclear pores were identified as breaks in the nuclear envelope (arrowheads). (Right) The perpendicular distance from the central plane of an NPC to a gold particle was measured and plotted in a histogram for each strain. The total number of gold particles and mean distance are given. Bar, 100 nm.

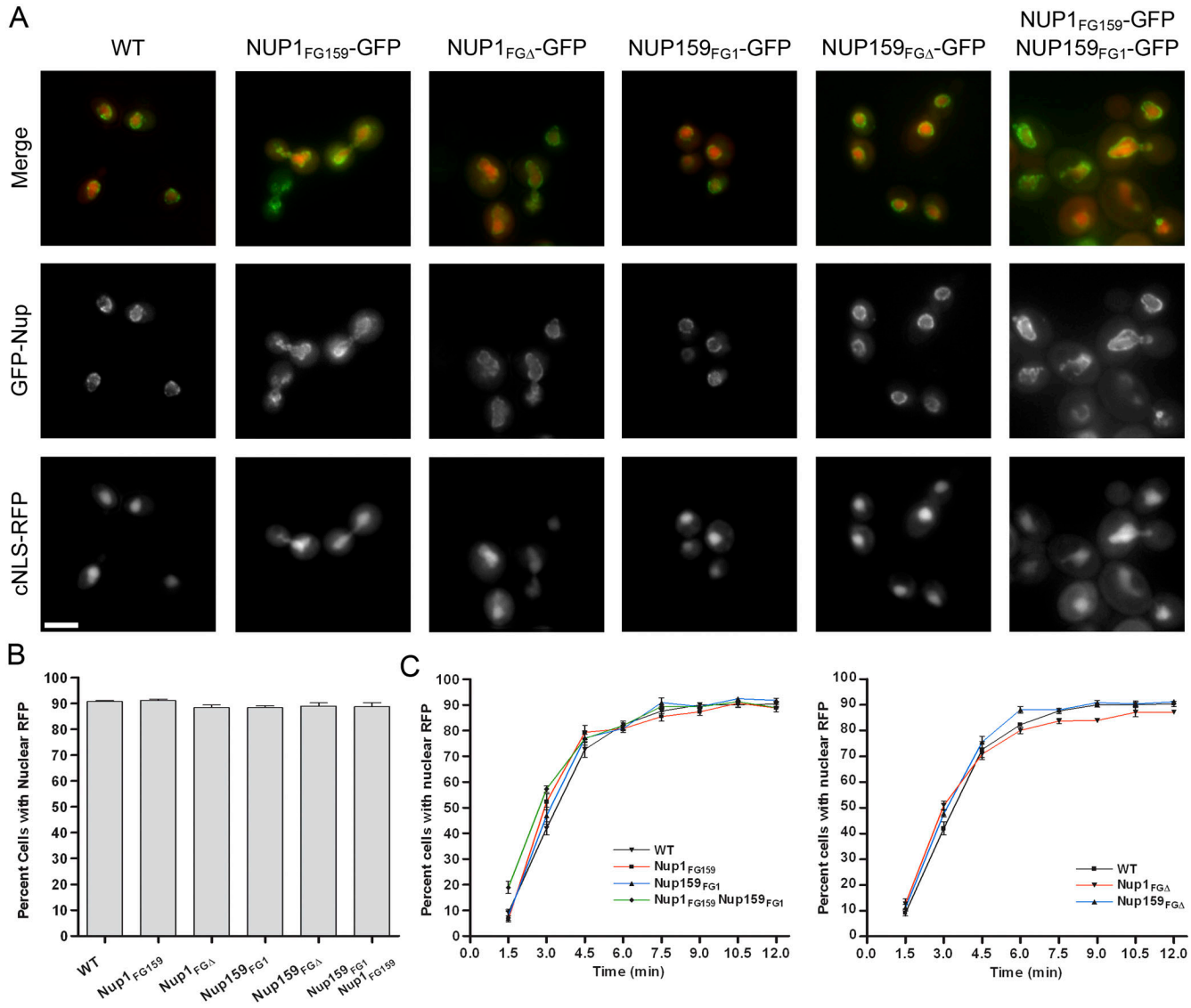
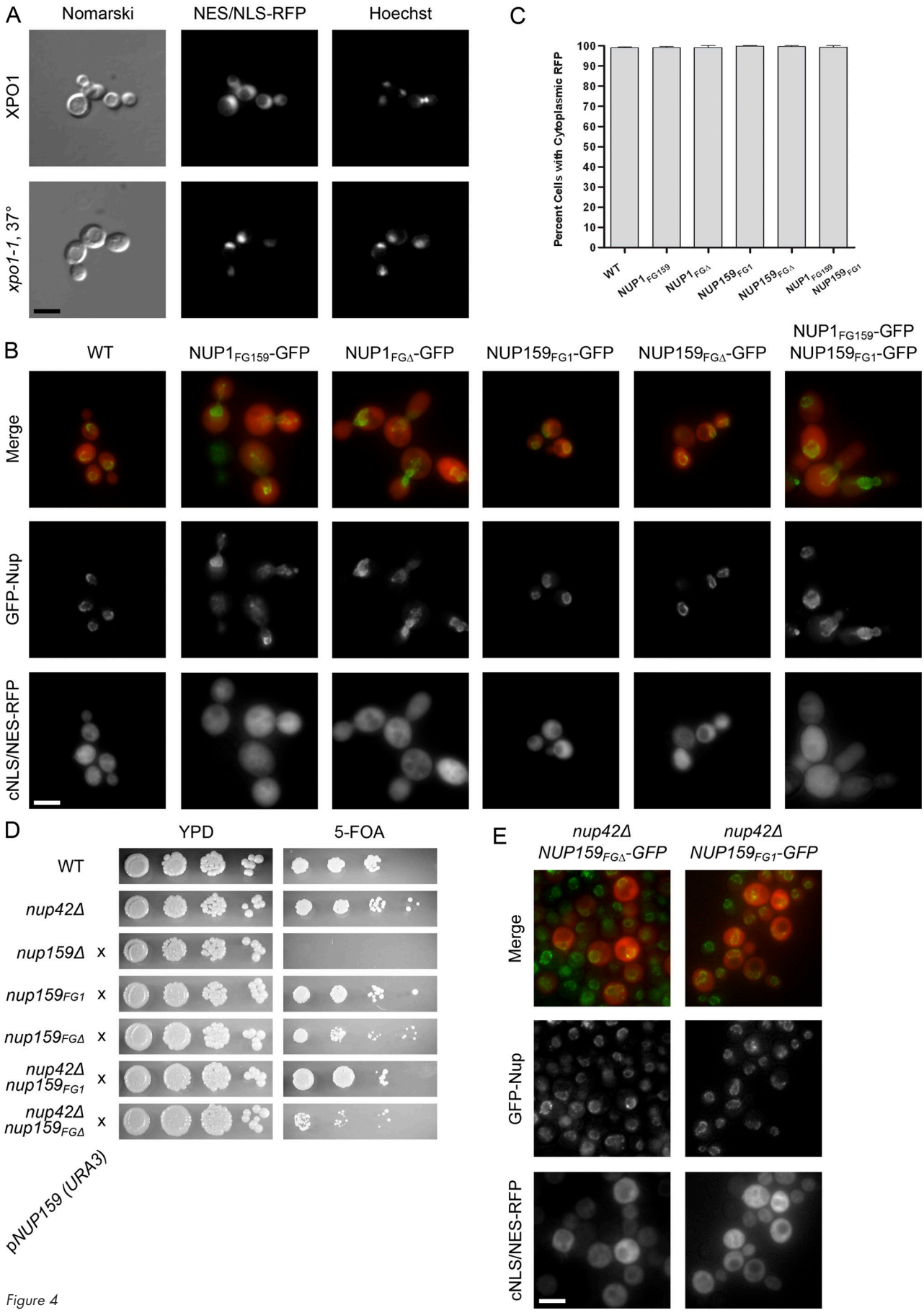



Figure 3. **The Kap95 import pathway is not affected in the FG mutants.** (A) Log-phase cultures were induced to express the cNLS-RFP reporter with 2% galactose and visualized after 2–4 h by fluorescence microscopy. Bar, 5 μm. (B) Quantitation of cNLS localization shown in A. A minimum of 300 cells were counted four independent times. The mean and SEM are presented. (C) Each strain from A was induced to express the cNLS reporter, metabolically poisoned, washed, and rescued with 2% dextrose. Recovery of nuclear RFP enrichment was then scored for at least 60 cells every 90 s. Each time point represents the mean and SEM of four experiments.

### Change of steady-state interactions between transport factors and the FG-swap Nups

Solution binding assays and pull-down experiments from whole cell extract have shown that transport receptor–cargo complexes have the highest binding affinity for Nups located nearest to their destination compartment. For example, importin β and its yeast orthologue Kap95 have a strong preference for Nups that are in the nuclear basket versus those localized to the cytoplasmic NPC surface or within the central channel (Allen et al., 2001; Ben-Efraim and Gerace, 2001; Pyhtila and Rexach, 2003). Conversely, Nup159 is the major NPC-binding partner of the export factor Xpo1/Crm1 within the NPC (Allen et al., 2002; unpublished data). Because biochemical and struc-

Figure 4. **Cytoplasmic FG repeats are not required for Xpo1-mediated export.** (A) Wild-type or *xpo1-1* cells were induced with 2% galactose for 3 h, stained with Hoechst dye, and visualized by fluorescence and bright field microscopy. *xpo1-1* cells were shifted to 37°C for 30 min immediately before microscopy. Bar, 5 μm. (B) Early-log phase cultures were induced to express the cNLS/NES-RFP reporter as in A and visualized by fluorescence microscopy. Bar, 5 μm. (C) Quantitation of cNLS/NES localization shown in B. A minimum of 300 cells were counted four independent times. The mean and SEM are presented. (D) Overnight cultures were grown to stationary phase in rich media and titered onto YPD (left) or 5-fluoroorotic acid (5-FOA). An "x" in the pNUP159 column denotes strains that harbored *NUP159-GFP* on a *URA3*-marked plasmid. (E) *nup42Δnup159*$_{FG1}$ and *nup42Δnup159*$_{FGΔ}$ cells containing the cNLS/NES-RFP reporter were examined for export defects as described for B. Note that for all strains tested, a large percentage of the cell population consistently failed to express the cNLS/NES reporter. Bar, 5 μm.


A
Nomarski
NES/NLS-RFP
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Percent Cells with Cytoplasmic RFP
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*Figure 4*

tural studies have established that the interaction between nuclear transport factors and Nups is directly mediated by the FG repeats, we predicted that swapping the FG domains between Nup1 and Nup159 would change their binding preference for Xpo1 and Kap95. To test this possibility, Xpo1 and Kap95 were fused to a carboxy-terminal ZZ tag and immunoprecipitated to assess their association with the Nup1 and Nup159 FG alleles (Fig. 2).

When Xpo1-ZZ was purified from yeast extracts, a strong interaction with Nup159, but not Nup1, was observed (Fig. 2 B, right). However, when the FG repeats of Nup159 were replaced with those from Nup1 ($Nup159_{FG1}$), the interaction between Nup159 and Xpo1 was virtually abolished. Furthermore, replacing the FG domain of Nup1 with that from Nup159 ($Nup1_{FG159}$) increased the binding of Xpo1 and Nup1 to levels similar to that observed for wild-type Nup159 (Fig. 2 B, right). Because $Nup1_{FG159}$ properly localizes to the nuclear side of the NPC (Fig. 1 D), we have thus generated an artificial "high affinity" binding site for Xpo1 at the nuclear face of the pore.

We also performed similar experiments using ZZ-tagged Kap95. Unexpectedly, we found that both Nup1 and Nup159 consistently associated with roughly equal amounts of Kap95 (Fig. 2 A, right). These interactions were unaffected by the presence of $Gsp1\Delta C_{Q71L}$-GTP, a known regulator of Kap95–Nup interactions (Allen et al., 2001; Maurer et al., 2001); yet $Gsp1\Delta C_{Q71L}$-GTP was able to dissociate Kap95 from Kap60 (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200407156/DC1). These data suggest that Kap95 can form stable complexes with Nups on both sides of the NE at steady state. This finding is in contrast to previous studies that have reported a difference in the affinities between Kap95 and cytoplasmic versus nuclear Nups (Allen et al., 2001; Denning et al., 2001; Pyhtila and Rexach, 2003). Notably, in our work, endogenous Kap95 was purified from total yeast extracts; whereas many of the previous experiments were performed using recombinant proteins or FG repeats in solution. Because we did not observe a significant difference in the strength of association between Kap95 and the FG Nups in our assay, we did not perform immunoprecipitations in the FG swap strains.

### The Kap95 and Kap121 import pathways are not affected in the FG-swap mutants

To address the functional consequence of altering the asymmetric FG repeats, we first examined Kap95-mediated protein import using a monomeric red fluorescent protein (RFP; Campbell et al., 2002) reporter fused to the classical NLS (cNLS). When expressed in WT cells, the cNLS-RFP cargo localized exclusively to the nucleus, outlined by Nup1-GFP staining (Fig. 3 A). Identical results were obtained for each of the FG-swap and -deletion strains (Fig. 3, A and B).

We also examined the rate of Kap95 import by treating cells with metabolic poison to allow leakage of the cNLS-RFP cargo into the cytoplasm, followed by recovery with glucose to enable the reimport of the reporter (Shulga et al., 1996). Importantly, the kinetics of import recovery for each of the FG mutants was indistinguishable from wild-type cells (Fig. 3 C), suggesting that the asymmetrically positioned FG repeats of Nup1 and Nup159 do not play a role in Kap95-mediated protein import.

We also tested the FG mutants for defects in the Kap121 import pathway using a GFP cargo fused to the minimal NLS of PHO4 (Kaffman et al., 1998). No significant difference was observed for the FG mutants compared to wild-type cells (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200407156/DC1). Furthermore, we were unable to detect a significant reduction in the rate of import of the PHO4-NLS reporter using the same kinetic analysis as described for cNLS import (Fig. S2 B).

### Cytoplasmic FG repeats are dispensable for NES export

Because the FG swap between Nup159 and Nup1 created an artificial binding site for Xpo1 in the nucleus (Fig. 2 B), we next sought to examine whether the FG mutants were deficient in Xpo1-mediated export. We took advantage of a highly sensitive in vivo export assay, which relies on the competition between a nuclear export signal (NES) and an NLS fused in cis to a reporter cargo (Stade et al., 1997). As expected, a cNLS/NES-RFP cargo was enriched in the cytoplasm of wild-type cells, but rapidly accumulated in the nucleus of *xpo1-1* mutants at the nonpermissive temperature (Fig. 4 A). When the reporter was expressed in the *nup1* and *nup159* FG mutants, no defect in its export was detected (Fig. 4, B and C). Because cNLS-mediated protein import is unaffected in all FG mutants tested (Fig. 3), we conclude that moving the high affinity binding site for Xpo1 from one side of the pore to the other does not significantly affect the export of NES-containing cargos.

Several FG Nups are not essential for viability in yeast, and it is expected that the FG repeats within the NPC exhibit some degree of functional redundancy (for reviews see Rout and Aitchison, 2001; Suntharalingam and Wente, 2003). In addition to Nup159, the nonessential Nup42 localizes to the cytoplasmic surface of the NPC and is known to interact with Xpo1 (Allen et al., 2001). To test whether Nup42 can compensate for the loss of the Nup159 FG repeats, we generated strains in which both *NUP159* and *NUP42* were mutated. Combinations of a *nup42*Δ with $nup159_{FG\Delta}$ or $nup159_{FG1}$ were viable and had growth rates similar to the single *nup159* FG mutants (Fig. 4 D). The double mutants also exported the NES reporter as efficiently as wild-type cells (Fig. 4, E and B). These results indicate that a high affinity cytoplasmic binding site for Xpo1 is not required for efficient protein export from the nucleus.

### mRNA export is not affected by altering the NPC FG-asymmetry

It is presently unclear how mRNAs are directionally exported through the NPC, because this transport pathway appears to function independently of the RanGTP gradient (for review see Reed and Hurt, 2002). We tested whether the correct positioning of the FG repeats of Nup1 and Nup159 is required for mRNA export by using an in situ hybridization probe to poly(A) mRNA (Fig. 5). As expected, mRNA export was in-

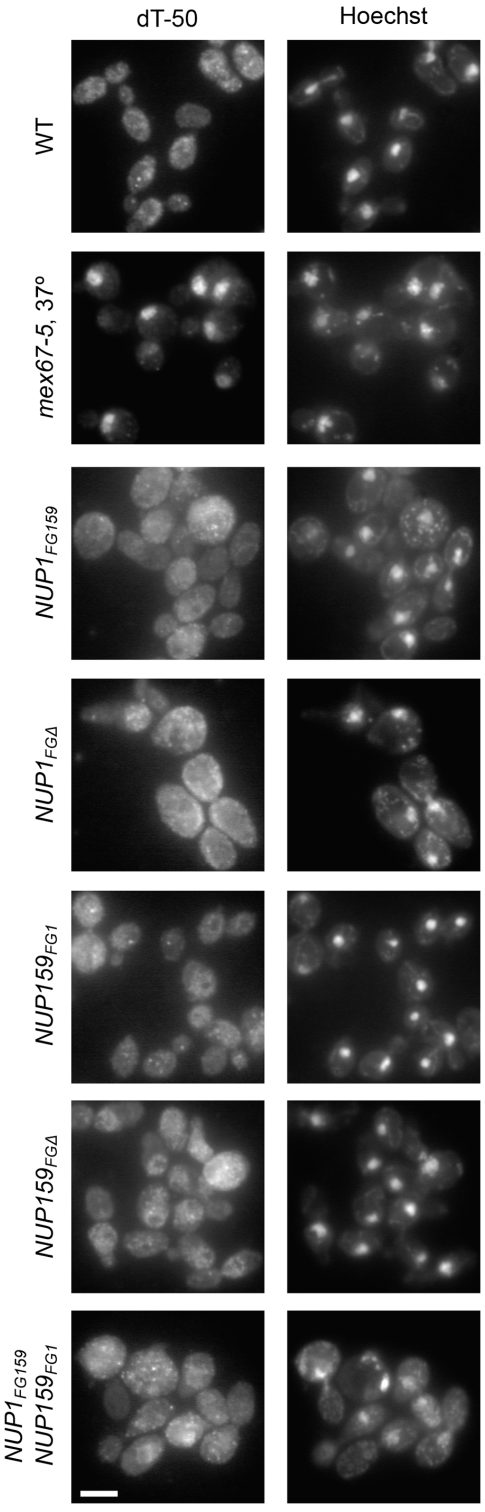


Figure 5. **poly(A) RNA export is not inhibited in the FG mutants.** Log-phase cells were fixed and stained for poly(A) RNA using an oligo-d(T) probe and detected by indirect immunofluorescence. DNA was labeled with Hoechst dye to visualize nuclei. *mex67-5* cells were shifted to 37°C for 1 h before fixation. Bar, 5 μm.

hibited in the export mutant *mex67-5* at the nonpermissive temperature. In contrast, no nuclear accumulation of poly(A) RNA was detected in wild-type cells or in any of the *nup1* or *nup159* FG mutants. Therefore, we conclude that the FG-asymmetry of Nup1 and Nup159 is not required for efficient mRNA export.

Together, our data strongly suggest that the asymmetric FG repeats of Nup1 and Nup159 are not required for Kap95- and Kap121-mediated import, Xpo1-mediated export, or mRNA export. However, because we have only analyzed the FG-repeats, we cannot exclude the possibility that additional high affinity binding sites outside these domains contribute to the efficiency of these import pathways (Pyhtila and Rexach, 2003). Nevertheless, our results are consistent with previous in vitro studies in *Xenopus* extracts demonstrating that the cytoplasmic filaments of the NPC are not essential for nuclear transport (Walther et al., 2002), because we have shown that an NPC without any cytoplasmic FG repeats (*nup42*Δ *nup159*$_{FG\Delta}$) is viable and fully functional for all transport pathways examined.

Our experiments also highlight the large degree of redundancy that exists within the pore. While this paper was in revision, a study from the Wente laboratory specifically addressed the question of FG-repeat redundancy. Intriguingly, more than half of the mass of the FG domains can be deleted without affecting viability, and strains lacking all five asymmetrically positioned FG-repeats are viable and have only modest transport defects (Strawn et al., 2004). We also attempted to generate such mutants, except we were unable to obtain viable *nup60*$_{FXF\Delta}$ *nup1*$_{FG\Delta}$ cells in our strain background. Although we are currently unable to resolve this discrepancy, it is important to note that our *nup1*$_{FG\Delta}$ deletion allele comprises a larger FG repeat region than the one used in the Wente study.

Finally, our data are in agreement with transport models suggesting that the NPC acts as a specificity gate that facilitates diffusion of certain cargoes, but does not directly impart directionality to the process of nucleocytoplasmic transport (Nachury and Weis, 1999; Ribbeck and Gorlich, 2001; Yang et al., 2004). Together, these studies provide strong evidence that unidirectional transport across the NPC does not depend on a gradient of FG repeats, but rather on the asymmetric distribution of non-NPC components, such as RanGTP.

## Materials and methods

**Extract preparation and immunoprecipitation**

1-liter cultures were grown to mid- to late-log phase and harvested for 10 min at 11,000 *g*. The pellets were washed with water, pelleted, and resuspended in an equal volume of 2× extraction buffer (EB; 60 mM Hepes, pH 7.3, 200 mM potassium acetate, 10 mM $MgCl_2$, 20% glycerol, 2 mM DTT, 10 mM GTP) supplemented with 2 mM PMSF and one third of an EDTA-free protease inhibitor cocktail tablet (Roche Applied Sciences). The suspension was immediately passed through a French press (Aminco) twice at 1000 PSI, and then pelleted for 20 min at 20,000 *g*. For each immunoprecipitation, 4 mg of extract was preincubated with Gsp1ΔC$_{Q71L}$-GTP or mock EB, then added to 50 μl of equilibrated IgG-Sepharose beads and incubated for 2–3 h. The beads were harvested at 400 *g* and washed three times for 10 min with 1 ml EB containing 50 mM NaCl. Finally, the beads were resuspended in reducing SDS-loading buffer, boiled, and loaded onto 8% SDS-PAGE gels. The separated proteins were transferred to a nitrocellulose membrane and blotted using monoclonal (for pull downs; Roche Applied Sciences) or polyclonal (for extracts; Seedorf et al., 1999) anti-GFP antibodies at 1:500 or 1:333, respectively. Anti–mouse or –rabbit secondary antibodies (Amersham Biosciences) were used at 1:2,500 and detected by ECL. The Kap60 and Kap95 antibodies were gifts from M. Rexach (Stanford University, Stanford, CA) and used at 1:1,000 and 1:3,000, respectively. The Xpo1 antibody was used at 1:10,000 (Maurer et al., 2001).

**Online supplemental material**

Detailed information regarding plasmid and strain construction, cell culture, fluorescence and electron microscopy, protein import assays, and in situ hybridizations is provided online as supplementary information. Fig. S1 demonstrates that Gsp1$\Delta C_{Q71L}$-GTP can dissociate Kap60 from Kap95 in extracts. Fig. S2 shows that the Kap121 import pathway is unaffected in the mutant FG strains. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200407156/DC1.

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