Distinct functions of the Drosophila Nup153 and Nup214 FG domains in nuclear protein transport

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The phenylanine-glycine (FG)–rich regions of several nucleoporins both bind to nuclear transport receptors and collectively provide a diffusion barrier to the nuclear pores. However, the in vivo roles of FG nucleoporins in transport remain unclear. We have inactivated 30 putative nucleoporins in cultured Drosophila melanogaster S2 cells by RNA interference and analyzed the phenotypes on importin α/β–mediated import and CRM1-dependent protein export. The fly homologues of FG nucleoporins Nup358, Nup153, and Nup54 are selectively required for import. The FG repeats of Nup153 are necessary for its function in transport, whereas the remainder of the protein maintains pore integrity. Inactivation of the CRM1 cofactor RanBP3 decreased the nuclear accumulation of CRM1 and protein export. We report a surprisingly antagonistic relationship between RanBP3 and the Nup214 FG region in determining CRM1 localization and its function in protein export. Our data suggest that peripheral metazoan FG nucleoporins have distinct functions in nuclear protein transport events.

Introduction

Yeast and vertebrate nuclear pore complexes (NPCs) are structurally similar and consist of multiple copies of ~30 different nucleoporins (Rout et al., 2000; Cronshaw et al., 2002). Approximately one third of all nucleoporins (Nups) carry phenylalanine-glycine (FG) repeats of variable length. They are found at the nuclear basket, cytoplasmic fibers, and the central part of the NPC and can bind to both importins and exportins (Tran and Wente, 2006). X-ray crystallography has mapped the contact sites between FG repeats and importin β, and mutations altering these amino acids in importin β also reduce nuclear protein import (Bayliss et al., 2002). The extended conformation of the FG regions, their abundance in the NPC, and their differential affinity for transport receptors suggest that they are major determinants of transport through the channel. However, genetic and biochemical experiments in yeast show that half of the FG repeats can be removed without any defect in protein transport and cell viability (Strawn et al., 2004).

The FG domain nucleoporins collectively provide a diffusion barrier to the pore. According to the virtual gating model, macromolecules are excluded from the pore by the fluctuations of unfolded peripheral FG domains. The local interaction between transport receptors and peripheral FG repeats traps the cargo, increases its residence time, and facilitates passage through the pore (Rout et al., 2003). In the selective phase partitioning model, intermolecular hydrophobic interactions between the FG repeats create a selective permeability barrier that prohibits free diffusion through the NPC. The interaction of nuclear transport receptors with distinct FG nucleoporins locally breaks the mesh and allows passage through the NPC (Frey et al., 2006). Are the mechanistic functions of all FG nucleoporins the same? Do individual metazoan FG nucleoporins contribute to protein transport differently than their yeast counterparts? We addressed these questions by functional analysis of the NPC using inducible GFP transport reporters in conjunction with RNAi in Drosophila melanogaster S2 cells.

Results and discussion

An RNAi screen for nucleoporin function in protein transport

We established inducible S2 cells expressing GFP, GFP fused to a classic NLS (cNLS [cNLS-GFP]), or GFP carrying a nuclear export signal (NES [GFP-NES]). Living cells expressing native GFP showed a homogenous distribution of the fluorescent signal (Fig. 1 A). The cNLS-GFP reporter accumulated in nuclei, whereas the GFP-NES cargo was localized predominantly in the cytoplasm (Fig. 1 A).

We tested whether the cNLS-GFP and GFP-NES reporters are cargoes of importin α/β and CRM1. We first treated
the cell lines with double-stranded RNA (dsRNA) against the
*Drosophila* homologues of importin α1, α2 (pendulin), α3, β
(ketel), or kapβ3 (Malik et al., 1997; Lippai et al., 2000). Only
the addition of importin α3 and β dsRNAs reduced the relative
levels of nuclear cNLS-GFP. The distribution of the GFP
and GFP-NES reporters was unaffected by the dsRNA treat-
ments (Fig. S1 A, available at http://www.jcb.org/cgi/content/
full/jcb.200612135/DC1; and not depicted). Thus, the cNLS-
GFP reporter is transported into the nucleus by importin α3/β.
In parallel, we treated the reporter cell lines with dsRNA for
CRM1 (emb; Collier et al., 2000). The nuclear intensity of
GFP-NES was increased in CRM1-depleted cells. This pheno-
type was comparable with the one generated by the treat-
ment of GFP-NES cells with leptomycin, a CRM1-specific
inhibitor (Fig. S1 B). Therefore, the cytoplasmic accumula-
tion of GFP-NES provides a functional assay for CRM1-
mediated export.

To assess the relative contributions of the NPC compo-
nents on cNLS import and NES export, we searched the
*Drosophila* genome database for nucleoporins. We identifi-
ed a set of 30 putative nucleoporins and a protein export cofactor,
RanBP3, in *Drosophila* (Table S1, available at http://www.jcb.
org/cgi/content/full/jcb.200612135/DC1). We did not detect
any Pom121 and Nup180 homologues in the fly genome. The
putative nucleoporin function of the selected *Drosophila* genes
was also predicted by the Inparanoid algorithm (O’Brien et al.,
2005), which classified them as orthologues of human genes
encoding nucleoporins (Table S1). For simplicity, we will refer
to the putative *Drosophila* nucleoporins by the names of their
human homologues.

We generated dsRNAs targeting each candidate nucleo-
porin and tested gene inactivation efficiency in the reporter
cell lines by RT-PCR and by immunostainings and Western
blots in cases in which specific antibodies were available
(Fig. S1, C–E). The dsRNA treatments considerably reduced
the endogenous gene product after 4 d and allowed functional
analysis of the genes in protein transport. The cellular distribu-
tion of each GFP reporter was assessed in parallel 4 d (Table S1)
and 6 d (unpublished data) after the addition of dsRNA to the
cultures. To avoid artifacts as a result of the potential off-target
effects of the dsRNAs, we generated a second set of dsRNAs
for all nucleoporins that scored positive in the primary screen.
These dsRNAs generated similar defects in the distribution
of the reporters, arguing for phenotype specificity (see below
and Fig. S2, A–C; available at http://www.jcb.org/cgi/content/
full/jcb.200612135/DC1).

![Figure 1.](http://www.jcb.org/cgi/content/full/jcb.200612135/DC1)

Figure 1. cNLS-GFP import defects in Nup358, Nup153, and
Nup54 RNAi cells. (A) Localization of GFP, cNLS-GFP, and GFP-
NES in S2 cells. Hoechst staining visualizes nuclei. (B) Cells
expressing cNLS-GFP were treated with importin β, Nup358,
Nup153, or Nup54 dsRNAs. (C) Ratios of nuclear to cytoplasmic
cNLS-GFP (left) and GFP-NES (right) intensities in untreated and
RNAi cells. dsRNA treatments reduced the nuclear accumulation of
cNLS-GFP compared with untreated cells (P < 0.0001 by pair-wise
t test). GFP-NES distribution was only affected in CRM1 cells (P > 0.05
by pair-wise t test for the nucleoporins). Error bars indicate SD.
30–35 cells were quantified for each treatment. Bars, 5 μm.
Selective requirement of Nup358, Nup153, and Nup54 in cNLS-GFP import

Cells treated with dsRNAs for Nup358, Nup153, or Nup54 exhibited a clear reduction in cNLS-GFP nuclear concentration but showed no defects in GFP-NES and GFP localization, suggesting a selective role for Nup358, Nup153, and Nup54 in cNLS-protein import (Fig. 1, B and C; Fig. S2 D; and Table S1).

The import phenotype might be secondary to structural defects in the NPC caused by silencing of the nucleoporin genes. To assess NPC integrity, we stained dsRNA-treated cells with the nucleoporin marker mAb414 and a panel of specific antibodies against NPC components: Nup214 and Nup88 at the cytoplasmic face (Fornerod et al., 1997), gp210 at the central core (Wozniak and Blobel, 1992), and TPR in the nuclear basket (Cordes et al., 1997). We found a pronounced reduction in mAb414 and anti-TPR rim labeling in Nup153 dsRNA-treated cells (Fig. 2). In addition, a substantial amount of Nup214 and its binding partner Nup88 was displaced from the pore (Fig. 2). Thus, both the cytoplasmic and nuclear basket nucleoporins are severely affected in Nup153 RNAi cells. We did not detect any phenotype with the gp210 antibody, suggesting that this part of the central core was intact (Fig. 2). None of the NPC composition defects in Nup153-depleted cells were detected in cells lacking Nup358 or Nup54, arguing that the import deficiency in these cells was not caused by major changes in pore integrity.

We further examined whether the RNAi inactivations caused defects in the localization or the amount of importin β by in situ stainings and Western blots (Fig. 3, A and B). Untreated cells showed the characteristic rim-staining pattern of importin β. Nup358 RNAi cells exhibited a weak cytoplasmic staining. The importin β signal was also reduced in Nup54 dsRNA-treated cells, but its localization was not affected. In Nup153 RNAi cells, the levels of importin β were not appreciably affected, but a substantial fraction of the protein was displaced from the rim.
into the cytoplasm (Fig. 3, A and B). Thus, in all cases, the nuclear import deficit of the dsRNA-treated cells correlates with defects in the levels and/or localization of importin β. Neither the distribution nor the intensity of CRM1 staining was appreciably changed in these cells (Fig. S2 E), implying that Nup358, Nup153, and Nup54 are selectively required for importin β–mediated import. Our genetic analysis of Nup153 and Nup54 function in cNLS import is consistent with studies in yeast (Nup57; Bucci and Wente, 1998), Xenopus laevis oocytes (Nup153 [Walther et al., 2001] and Nup54 [Finlay et al., 1991]), and HeLa cells (Nup153; Shah and Forbes, 1998) using immunodepletion and over-expression experiments. However, the role of Drosophila Nup358 is surprising. Nup358 is the major component of the cytoplasmic filaments, and immunodepletion of its Xenopus homologue does not cause cNLS import defects in oocyte nuclei (Walther et al., 2002). Drosophila Nup358 is essential for importin β
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expression or integrity (Fig. 3, A and B), and the cNLS-GFP mislocalization in nup358i cells may be caused by the massive reduction of importin β levels.

The FG-rich region of Nup153 is required for its function in cNLS import

A common feature of Nup358, Nup153, and Nup54 is the high content of FG repeats in their primary sequence. Does the FG-rich part of Nup153 contribute to nuclear import? To address this question, we overexpressed a V5-tagged full-length (V5-Nup153) and a truncated form of Nup153 lacking the FG domain (V5-Nup153 ∆FG) in Nup153 RNAi cells. Both chimeric proteins were expressed at similar levels and became localized at the nuclear envelope (Fig. 3, C and D). The full-length form restored both the pore composition defects and the cNLS-GFP phenotype (Figs. 3, C–E and S2, F–H), indicating that Drosophila Nup153, like its vertebrate homologues, contributes to both pore integrity and importin β transport (Walther et al., 2001). The Nup153 ∆FG fragment could rescue the defects in Nup214 and TPR localization in >98% of the expressing cells (n = 79), suggesting that it contains all of the necessary sequences for Nup153 function in NPC integrity (Figs. 3 C and D). We examined whether the Nup153 ∆FG fragment is also sufficient to restore the importin β localization and cNLS import defects of Nup153 dsRNA-treated cells. In 50% of the V5-positive cells (n = 82), importin β accumulation resembled its steady-state localization in untreated cells (Fig. 3 C, bottom), suggesting that the Nup153 FG repeats are partly redundant for importin β localization. Restoration of the import receptor at the NPC may be caused by the reinstatement of other importin β–binding FG nucleoporins like Nup214 (Xylourgidis et al., 2006). However, only 10% of the transfected cells (n = 66) displayed an increased nuclear cNLS-GFP accumulation (Fig. 3, D and E). The results argue that the role of Nup153 in protein import is independent of its function in NPC integrity. The FG region is required for importin β–mediated transport, whereas the remainder of the protein ensures an intact NPC. A direct role of the Nup153-FG part in conveying importin α3/β cargos through the pore is further supported by its localization along the entire channel (Fahrenkrog et al., 2002) and by its highly flexible conformation (Lim et al., 2006).

Antagonistic roles of Nup214 and RanBP3 in CRM1-mediated protein export

None of the dsRNA treatments against nucleoporins caused detectable defects in GFP-NES distribution (Table S1). However, the inactivation of RanBP3 increased the nuclear accumulation of the export reporter (Fig. 4, A and B). The treatment had no effect on GFP and cNLS-GFP localization (Table S1 and Fig. S3 A, available at http://www.jcb.org/cgi/content/full/jcb.200612135/DC1). Yrb2, the yeast homologue of RanBP3, is also essential for CRM1-dependent export (Taura et al., 1998; Noguchi et al., 1999). Vertebrate RanBP3 forms complexes with CRM1, RanGTP, and export substrates to stimulate NES nuclear protein export

Figure 4. Antagonistic functions of RanBP3 and Nup214 in GFP-NES export and CRM1 localization. (A) GFP-NES–expressing cells were treated with CRM1, RanBP3, or Nup214 dsRNAs. Hoechst staining visualizes nuclei. (B) Ratios of nuclear to cytoplasmic GFP-NES in untreated cells and cells subjected to CRM1, RanBP3, Nup214, or Nup88 dsRNA treatment. CRM1 and RanBP3 RNAi cells showed the increased nuclear accumulation of GFP-NES compared with untreated cells (P < 0.0001 by pair-wise t test). Nup214 and Nup88 RNAi did not significantly alter GFP-NES distribution (P > 0.05 by pair-wise t test). Error bars indicate SD. 30–35 cells were quantified for each treatment. (C) Cells treated with RanBP3 dsRNA, Nup88 dsRNA, Nup214 dsRNA, or a combination of RanBP3 and Nup214 dsRNAs stained for CRM1 (red) and mAb414 (green). (D) Cells treated with RanBP3 dsRNA or dsRNAs against both RanBP3 and the 3′ untranslated region of Nup214 (ranbp3i + nup214i-2) were transfected either with V5-Nup214 or V5-Nup214ΔFG plasmids. Cells were stained for V5, Nup88, and CRM1. The right column shows GFP-NES cells treated as in other columns stained for V5 and analyzed for GFP localization. V5 staining visualizes the expression of Nup214 fusion protein. In all cases, V5 labeling is presented in green. Bars (A), 5 μm; (C and D) 2.5 μm.
(Englmeier et al., 2001; Lindsay et al., 2001). RanBP3 and CRM1 were also found in complex with the chromatin-associated protein RanGEF (Nemer gut et al., 2002). We asked whether RanBP3 inactivation impacts CRM1 distribution by staining for CRM1. Untreated cells showed a predominantly nuclear accumulation of CRM1 with only a small fraction of the protein localized at the nuclear envelope (Figs. 4 C and S3 C). The nuclear CRM1 staining was severely reduced in RanBP3 RNAi cells. Instead, CRM1 became highly concentrated at the rim and, to some extent, in the cytoplasm of RanBP3 dsRNA-treated cells (Figs. 4 C and S3 C). The treatment had no effect on the accumulation of Nup214, Nup88, or any of the tested nucleoporins (Fig. S3 B), suggesting a new function of RanBP3 in CRM1 localization. Reexpression of V5-tagged RanBP3 at low levels in ranBP3i cells restored both CRM1 depletion from the nucleus and the NES export defect (Fig. S3, E and F). The results suggest that RanBP3 directly controls CRM1 localization and protein export.

CRM1 forms complexes with Nup88 and Nup214 (Fornerod et al., 1997; Roth et al., 2003), and, in Drosophila mutants lacking either of the nucleoporins, the NPC-bound CRM1 fraction accumulates in the nucleus (Roth et al., 2003; Xylourgidis et al., 2006). To determine whether Nup88 or Nup214 silencing causes similar phenotypes in S2 cells, we stained cells treated with Nup88 or Nup214 dsRNA for CRM1. The treatments reduced the CRM1 signal intensity at the nuclear envelope (Figs. 4 C and S3 C), suggesting that Nup88 and Nup214 anchor a CRM1 fraction at the NPC of S2 cells. However, unlike the defects of nup88 (mbo) and nup214 mutant larvae, the inactivation of Nup214 or Nup88 in S2 cells did not increase the cytoplasmic accumulation of the GFP-NES reporter (Fig. 4 A). Thus, Nup214 or Nup88 depletion has no impact on CRM1 activity in S2 cultured cells. This difference between larval tissues and S2 cells can be attributed to the relatively high levels of CRM1 bound to the NPCs of distinct larval tissues (Uv et al., 2000; Roth et al., 2003; Xylourgidis et al., 2006). The redundancy of Nup214 for NES-GFP export in S2 cells is consistent with the lack of detectable defects in the nuclear export of NLS-GFP-NEs in HeLa cells depleted for Nup214 (Bernad et al., 2006). Surprisingly, RNAi inactivation of Nup214 in the same cell line resulted in defects in nuclear export of the NFAT (nuclear factor of activated T cells) transcription factor and, to a lesser extent, in export of the Rev-GR-GFP reporter (Hutten and Kehlenbach, 2006). The different phenotypes may suggest specific requirements of the different export cargoes used in the two studies.

CRM1 can shuttle between the nucleus and the cytoplasmic face of the NPC in an energy-independent manner (Becskei and Mattaj, 2003), and the inactivation of Nup214 and RanBP3 show opposing phenotypes in its localization. Therefore, we investigated CRM1 accumulation in cells treated simultaneously with both Nup214 and RanBP3 dsRNAs. In these cells, CRM1 was found inside the nucleus (Fig. 4 C), suggesting that RanBP3 and Nup214 antagonize each other to determine the nuclear concentration of CRM1. The C-terminal FG-rich region of Nup214 binds to CRM1 directly (Xylourgidis et al., 2006), and we asked whether it is also required for its antagonistic role in CRM1-mediated export. We expressed V5-tagged full-length or FG-deleted versions of Nup214 in cells lacking both RanBP3 and Nup214, where CRM1 accumulates inside the nucleus. The V5-Nup214 protein complemented the Nup88 defect at the nuclear envelope (Fig. 4 D, middle column) and prohibited the nuclear accumulation of CRM1 (Fig. 4 D, left column). The V5-Nup214ΔFG protein was expressed at similar levels as the wild-type protein and rescued the Nup88 degradation defect
caused by the Nup214 inactivation (Fig. 4 D; Xylourgidis et al., 2006). Thus, the N-terminal part of Nup214 is sufficient for the interaction with Nup88 and NPC. However, the V5-Nup214ΔFG fragment only slightly increased the NPC-bound fraction of CRM1 (Fig. 4 D). This small amount of CRM1 at the rim may be attracted by Nup88, which also binds to the export receptor (Roth et al., 2003). The results suggest that the antagonistic function of Nup214 on CRM1 localization is dependent on the Nup214 FG repeats.

How do the opposing roles of Nup214 and RanBP3 on CRM1 accumulation influence its activity in NES export? Treatment of GFP-NES–expressing cells with dsRNAs against both Nup214 and RanBP3 resulted in the cytoplasmic distribution of the reporter, closely resembling its accumulation in untreated cells (Fig. 4 D, right column). Thus, unleashing the pore-bound fraction of CRM1 through Nup214 inactivation largely restores the GFP-NES export defect caused by the depletion of RanBP3. The results suggest that CRM1 NES export activity can be tuned by the opposing functions of Nup214 and RanBP3. Overexpression of the full-length Nup214 construct in Nup214 and RanBP3 RNAi cells resulted in a nuclear accumulation of GFP-NES closely resembling the phenotype caused by single RanBP3 inactivation (Fig. 4 D). In contrast, the distribution of GFP-NES remained unaffected in V5-Nup214ΔFG–expressing cells lacking both Nup214 and RanBP3 (Fig. 4 D). The results indicate that the ability of Nup214 to antagonize the function of RanBP3 in NES export requires the Nup214 FG repeats.

CRM1 is reduced in the nucleus of RanBP3i cells, arguing that RanBP3 retains it inside the nucleus. To further examine the proposed new role of RanBP3, we overexpressed it in both cells and analyzed its effects on CRM1 and GFP-NES localization. The expression of V5-tagged RanBP3 increased the nuclear intensity of CRM1 (Figs. 5 A and S3 D), further arguing for a dynamic equilibrium between the Nup214- and RanBP3-bound forms of CRM1 (Fig. 5 C). In parallel experiments, we assessed the effect of RanBP3 overexpression in GFP-NES distribution. Although low levels of V5-RanBP3 did not change the predominantly cytoplasmic distribution of GFP-NES, high amounts of the exogenous protein increased the nuclear intensity of the reporter (Fig. 5 B). This phenotype is consistent with in vitro experiments in which high levels of RanBP3 inhibit the assembly of CRM1 export complexes. In summary, we propose a dual function of RanBP3 (Fig. 5 C): one maintaining high nuclear levels of CRM1 and one aiding the assembly of CRM1–RanGTP cargo complexes (Englmeier et al., 2001; Lindsay et al., 2001).

In our vivo analysis of nucleoporin function by RNAi we did not detect protein transport phenotypes for the majority of the nucleoporins. This could be the result of functional redundancy, incomplete gene inactivation, or the relatively insensitive reporter assays. Nevertheless, the data provide some new insights into the function of NPCs.

First, Nup214 and RanBP3 antagonize each other to determine CRM1 localization and function. RanBP3 has a primary role in maintaining CRM1 inside the nucleus. This function of RanBP3 becomes redundant when Nup214 is codelleted. Second, we provide genetic evidence arguing that individual FG domains are essential for distinct transport pathways in Drosophila. The importance of the Nup153 FG motif in mediating cNLS import was already suggested by overexpression experiments in permeabilized HeLa cells (Shah and Forbes, 1998). Surprisingly, the FG repeats of Nup214 do not facilitate NES-GFP export but rather inhibit it. The FG regions from Nup153 or Nup214 are indispensable for the distinct transport roles of Nup153 and Nup214, yet they are not expected to affect the total mass of FG repeats and the barrier function of the NPC. The genetic analysis of nucleoporins in Drosophila argues that the Nup153 and Nup214 FG regions have specific functions in import and export, respectively, and suggest that peripheral nucleoporins have acquired additional roles during metazoan evolution. Understanding the mechanistic roles of animal nucleoporins in endogenous protein transport may provide new insights into the regulatory potential of the NPC.

Materials and methods

Cell lines and cell culture

The GFP, cNLS-GFP, and GFP-NES constructs were described previously (Roth et al., 2003). S2 cells were cultured in Drosophila Schneider medium (PAN) supplemented with FCS, glutamine, and streptomycin/penicillin (Invitrogen). Stable cell lines were generated by hygromycin selection according to the manufacturer’s instructions (Invitrogen). The expression of GFP-tagged cargos was induced by 0.2 mM CuSO4 for 16 h. Leptomycin B was used at the concentration of 10 ng/ml for 15 min.

dsRNA treatments

RNAi was performed essentially as described previously (Clemens et al., 2000). Primer pairs tailed with the T7 RNA polymerase promoter were used to amplify PCR fragments obtained from cDNA clones. PCR products with an average size of 700 bp were then used as templates for dsRNA production with the MEGAscript RNAi kit (Ambion). For transfection, 15 μg dsRNA was added to 2.5 × 106 S2 cells in six-well plates.

Live cell imaging

DNA was visualized by the addition of Hoechst 33342 (Sigma-Aldrich) at a concentration of 4 μM. Images were recorded with an inverted fluorescence microscope (DM IRB; Leica) at days 4 and 6 after dsRNA treatment and were quantified using Velocity version 2.0.1 (Improvision).

Western blotting and RT-PCR

Cells were lysed in 10 mM Tris, 140 mM NaCl, 1.5 mM MgCl2, and 1% NP-40. Lysates were resolved by SDS-PAGE and analyzed by immunoblotting. Blots were developed using the ECL Advance kit (GE Healthcare). Images were acquired with a luminescent image analyzer (LAS1000; Fuji) and quantified with Image Gauge version 3.45 (Fuji). For RT-PCR, mRNAs were isolated using magnetic oligo(dt)-coupled beads (Dynabeads). Reverse transcription was performed with SuperScript II (Invitrogen).

Rescue experiments

The inactivation of Nup153 was performed with dsRNAs generated by the primers 5′-TATATCGACTCATAAGGAGGATGGTGTTGGAATTCCCC-3′ and 5′-TATATCGACTCATAAGGAGGATGGTGTTGGAATTCCCC-3′. Nup214 was inactivated with dsRNAs made by the primers 5′-TATATCGACTCATAAGGAGGATGGTGTTGGAATTCCCC-3′ and 5′-TATATCGACTCATAAGGAGGATGGTGTTGGAATTCCCC-3′. Nup214 was inactivated with dsRNAs generated by the primers 5′-TATACTGCTATATCGACTCATAAGGAGGATGGTGTTGGAATTCCCC-3′ and 5′-TATACTGCTATATCGACTCATAAGGAGGATGGTGTTGGAATTCCCC-3′. In all cases, products were targeting the 3′ untranslated region of the respective mRNAs. The cDNA encoding full-length Nup153 (amino acids 1–1,905) and Nup153ΔFG (amino acids 1–1,288) were introduced into the BstBII site of pAc5.1/V5-His (Invitrogen). The cDNAs encoding Nup214 (amino acids 1–1,670) and Nup214ΔFG (amino acids 1–1,080) were inserted into the NotI site of pAc5.1/V5-His. The cDNA encoding RanBP3 (amino acids 1–451) was inserted into the BstBII site of pAc5.1/V5-His.

Antibodies

We used antibodies against CRM1 (Roth et al., 2003), importin β (provided by J. Szabad, University of Szeged, Szeged, Hungary; Lippai et al., 2000),...
Nup88 (Uv et al., 2000), Nup214 (Roth et al., 2003), TPR (a gift from V. Cordes, University of Heidelberg, Heidelberg, Germany), α-tubulin (Sigma-Aldrich), mAb414 (Babco), lamin (provided by G. Krohne, University of Wurzburg, Wurzburg, Germany; Wagner et al., 2004), and gp210 (provided by P.A. Fisher, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA; Fison et al., 1985).

Immunofluorescence and confocal microscopy
S2 cells were attached to poly-L-lysine–(Sigma-Aldrich) or ConA (Sigma-Aldrich)-coated coverslips. Adherent cells were fixed with 4% PFA for 30 min in PBS, permeabilized for 5 min in 0.1% Triton X-100/PBS, and incubated overnight at 4°C with primary antibodies diluted in 0.5% BSA/PBS. Cells were washed in 0.1% Triton X-100/PBS, incubated with secondary antibody for 2 h at RT, and incubated with 0.4 mg/ml DAPI for 5 min. Cells were mounted in Vectashield (Vector Laboratories). Wide-field images were acquired with Openlab version 3.1.4 (Improvision), and ratios of nuclear envelope to cytoplasmic labeling intensities were quantified using Velocity version 2.0.1 (Improvision).

Confocal images were collected with a laser-scanning microscope (LSM510; Carl Zeiss Microimaging, Inc.) and processed with LSM510 software (Carl Zeiss Microimaging, Inc.). For quantitative analysis of confocal images, the nuclear membrane was reduced to a binary image. A distance transform function was applied, producing separate maps of distances into the nucleus and the cytoplasm. The distance maps were used to define regions of interest at increasing distances from the nuclear membrane. The nucleolus, areas outside the cell, vacuoles, and nuclear membrane folds were excluded from the analysis. The average concentration gradient for a cell population was obtained by giving equal weight to the individual concentration gradients. Software was based on a Semperów kernel (Syncroscopy).

Online supplemental material
Table S1 summarizes the transport phenotypes after dsRNA treatment against Drosophila nucleoporins. Fig. S1 shows importin α/β and CRM1 transport assays in Drosophila S2 cells (A and B) and shows the inactivation efficiency in dsRNA-treated cells (C–E). Fig. S2 shows the cNLS-GFP import defect in cells treated with the second set of Nup358, Nup153, and Nup54 dRNAs (A–C) and shows CRM1 and GFP-NES localization in cells defective in cNLS-GFP import (D and E). Fig. S2 also shows the restoration of TPR and Nup214 localization by full-length Nup153 or Nup153ΔFG (F–H). Fig. S3 shows the NPC integrity and cNLS-GFP import in RanBP3 RNAi cells (A–C) and shows CRM1 and GFP-NES localization in cells defective in cNLS-GFP import (D and E). Fig. S2 also shows the restoration of protein p270/Tpr as a constitutive component of the nuclear pore complex-associated intranuclear filaments. J. Cell Biol. 136:515–529.


